

REMARKS

According to the above amendments, claim 43 has been amended. Claims 43-67 remain in this application with claims 49 and 57-67 presently withdrawn from further consideration as being drawn to a non-elected invention. Thus, claims 43-48 and 50-56 are currently being examined. No claim has been allowed.

Withdrawal of the previous rejection of claims 43-48 and 50-56 under 35 USC § 112, second paragraph, is gratefully acknowledged.

New Claim Rejections - 35 USC § 112

Clarity

Claims 43-48 and 50-56 are newly rejected under 35 USC § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection has several facets.

In a first rejection, the Examiner has objected to the amendment of "*having a first cell binding activity*" in a first cell binding activity in claim 43(i). Accordingly, "in" has been amended back to having which should meet this rejection.

Mechanism of incorporation of passenger peptide into viral particle

In order to clean up some possible confusion with regard to claim wording indefiniteness and clarity, some additional

information regarding the mechanism of incorporation of the passenger peptide into the viral particle is offered. This represents an important aspect of the invention and the explanation may be helpful.

Thus, whereas the wording of claim 43 may, arguably, appear to suggest that the invention involves a defined active process of incorporation, in fact, the invention stems from an identification by the inventors that the passenger peptide is actually incorporated spontaneously into the viral particle when co-expressed. Claim 43(ii) has been amended to better reflect that the incorporation process proceeds on its own. The protein that the inventors used (SCF) has a naturally-occurring membrane-bound form and this is automatically inserted into the packaging cell plasma membrane. Applicant submits that any similar protein would be processed in the same way. The co-expression of the mb-SCF and the viral particle leads to insertion of mb-SCF into the plasma membrane of the packaging cell, which is then incorporated into the expressed viral particles through budding of the viral particles from the packaging cell.

Other passenger peptides may be made to act in the same way as mb-SCF. The inventors have successfully used the membrane anchor region of SCF to make a normally secreted protein, membrane bound. All membrane bound proteins carry a signal peptide at the N terminus that allows them to be inserted into the plasma membrane by the cellular protein processing machinery.

A signal peptide can be added artificially to any protein, if necessary. Applicant believes this to be well within the capabilities of a person skilled in the art. Thus, it follows that any passenger peptide with a membrane anchor will, when co-expressed with the viral particle, be spontaneously incorporated into the viral particle in the manner described by the present inventor's in the specification.

Origin of Passenger Peptide

In a second rejection under 35 USC § 112, second paragraph, directed to claim 43(ii), the Examiner has raised two issues. First, the Examiner has objected to the language in the previous amendment that reads, "and incorporates said passenger peptide binding moiety into said packaging into said packaging cell membrane". This is deemed unclear because, in the Examiner's view, one cannot be sure whether it is a result of the expression of the nucleic acid or whether it results from a different process.

The Examiner also has rejected the claims based on the view that the language "incorporating said passenger peptide binding moiety into said packaging cell" in claim 43(ii) necessitates that the passenger peptide is derived from the packaging cell. This is perceived as an inconsistency with the amendment that states that "said passenger peptide is other than one derived from the virus or the packaging cell". The Examiner has

concluded that it is unclear where the passenger peptide originates. This rejection is respectfully traversed.

There appears to be something of a misunderstanding of the claim regarding the above issues which may have been solved, at least in part, by the explanation in the descriptive material relating to the mechanism above which might be helpful in solving the inconsistency.

In addition, it is also noteworthy that an important aspect of the invention lies in the method of altering the binding activity of a viral particle by displaying surface proteins of choice on the viral particle surface. The current invention demonstrates that a naturally occurring cellular surface protein, not normally present on the surface of the retroviral packaging cells can be incorporated into the surface of retroviral particles.

The "passenger peptide" would normally be a protein not expressed by the fibroblast (HEL293 most commonly) used to package the retrovirus. This is incorporated into the cell by transfection using calcium phosphate or lipids (standard technology known in the art). The inventor has made use of plasmid vectors that allow easy establishment of stable transfectants (for lentiviral work transient transfection is used).

The specific surface protein of choice may differ according to the intended application of the modified viral particles.

This could be determined by the skilled person from an understanding of the teaching of the specification. A key advantage of the current invention is an ability to incorporate a molecule of choice into the surface of the viral particle. The skilled person would understand from the teaching of the specification that this molecule may be a naturally-occurring cellular surface protein, which is not a retroviral envelope or receptor. The origin of the passenger peptide would therefore depend on the cells to be targeted by the modified viral particles.

Enablement

The Examiner has maintained the previous rejection that claims 43-48 and 50-56 lack enablement commensurate with the claimed scope. The Examiner considers the specification to only be enabling for methods using mbSCF as passenger peptide and c-kit receptor expressing cells as target cells.

It appears that the main thrust of the rejection relates to the Examiner's position that the incorporation of a passenger peptide into the viral particle using the methods of the current invention is inherently unpredictable. With the exception of the worked example in the specification (mbSCF), the Examiner does not consider the method to be easily transferable for use with other peptides.

This rejection is respectfully traversed. Additional explanation and support for applicant's position are presented below.

Incorporation of membrane proteins into viral particle

Good evidence exists that the majority of proteins present on the cell surface become incorporated into retroviral particles. For example, see Hammarstedt et al. (2000) Minimal exclusion of plasma membrane proteins during retroviral envelope formation. PNAS USA. 97:7527-7532. also, Arthur et al (1992, Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science 258: 1935-1938). These publications are attached as Exhibit A and Exhibit B, respectively. They have shown that retroviruses made in cells from different strains of mice are immunologically distinct and have surface proteins characteristic of the specific host cell. Therefore, it follows that the incorporation of membrane associated peptides into budding viruses is eminently predictable. The membrane associated proteins that are incorporated into the virus particle will thus depend on the membrane proteins associated with the particular packaging cell and those intentionally expressed in that cell.

As we have previously stated, the present invention makes use of an envelope that cannot naturally bind to the target cells of interest. The experiments showed that after packaging with the passenger peptide, the viral particles only bound to target

cells that were expressing the specific cognate receptor of the passenger peptide. The Examiner has agreed that the viral particles of the examples exhibit the claimed properties. Applicant submits that the specification also supports and enables the position that the methods are transferable to other potential passenger peptides, as will be discussed.

Many studies using chimeric envelope proteins have demonstrated that peptides displayed in this way retain binding activity, e.g. single chain antibody additions to viral envelope. The inventors do not modify the "passenger peptide" from its native form. There is no evidence to suggest that any natural membrane bound protein would lose its binding capacity when incorporated into the virus particle. The Examiner has not provided any evidence to suggest the view that other binding proteins would not retain their binding capacity when incorporated into the viral particle. The skilled person would understand and appreciate that such proteins would retain their binding capacity.

The applicant has previously provided support showing that enveloped viruses behave in an almost identical manner when budding and therefore the mechanism of incorporation of proteins into the viral particle will be the same irrespective of the virus used. Thus, the behavior of other potential passenger peptides is believed to be sufficiently predictable.

As is well known, retroviruses can be pseudotyped with envelope proteins from many other enveloped virus types, e.g. VSV-G, SNV env, influenza haemagglutinin. Also, a retroviral envelope has been used with other virus types. Modifications to the vaccinia virus surface have also been made. In view of this, modification of viral particles with proteins from other viruses or with other membrane proteins is believed eminently achievable and the binding capacity of the resultant viral particles is predictable.

Properties of membrane proteins to be incorporated into viral particle

The Examiner has cited particular properties of the membrane proteins that would allegedly affect the predictability of the transferability of the methods to other proteins. These objections are all believed to have been addressed by the teaching of Hammarstedt et al (2000) which shows that virtually all plasma membrane proteins from the host cell are present in the retroviral particle.

The only requirement for retroviral incorporation appears to be that the protein resides in membrane structures known as "lipid rafts" (Esser et al. (2001) Differential incorporation of CD45, CD80 (7-1), CDS6 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type I virions and microvesicles: implications for viral

pathogenesis and immune regulation. J Virol. 75; 6173-6182)
(attached as Exhibit C).

Other concerns raised by the Examiner are addressed next in turn. The properties of the proteins in question are not believed to be particularly relevant and these properties would not affect their incorporation into the viral particle.

The Examiner has suggested that there may be a size limitation for incorporation of the membrane protein into the viral particle. There are no limits to the size of the protein that may be incorporated; the protein must simply reside in the cell membrane. Such proteins may be easily identified by the skilled person.

Other properties of the passenger peptide, such as charge, folding, post-translational modification, hydrophobicity, etc. would also have no relevance in terms of its incorporation into the viral particle membrane. The passenger peptide is expressed by the packaging cells and incorporated into the plasma membrane independent of the viral particle genome. The passenger peptide is then incorporated into the budding viral particle in a highly predictable manner irrespective of its properties.

Binding properties of membrane proteins

As demonstrated in applicant's mbSCF example, and previously indicated interactions between the passenger peptide binding moiety and other receptors or unintended proteins do not affect the claimed altered tropism. As discussed above, the inventor

does not modify the properties of the passenger peptide therefore its specific binding properties will be maintained. This is demonstrated in the examples by the failure to bind to c-kit negative cells. This is equally true of retroviruses with heavily engineered chimeric envelopes. Further, antibodies expressed on the surface of bacteriophages retain their specific binding capabilities. If the surface proteins are not modified there is no reason for them to lose their binding properties. The Examiner also has suggested that the growth conditions in which the viral particles are manufactured may affect the binding properties of the viral particles. No evidence is presented to support this statement. Retroviral particles are manufactured under normal physiological conditions of pH, temperature, ionic strength etc. These are clearly well known and also the conditions under which the viral particles would be used. Thus, this suggestion cannot properly be maintained.

The Examiner also suggests that interactions between the viral particles and target cells (or otherwise) that are independent of the specific passenger peptide/cognate receptor interactions may affect the viral particle tropism. The infectious titre of ecotropic virus on human cells is phenomenally low. This is generally below the limits of detection of most assays and has been well established in the art for about 30 years, Scholz et al (1996) Nucl. Acid Res 24 P979-980 (Exhibit D). Again, the Examiner has provided no evidence to

support this conclusion. Indeed, Chandreshekrnan et al (2004) cited by the Examiner, shows that transduction is directly dependent on the level of c-kit expression. Thus there was no non-specific binding with mb-SCF or through any mb-SCF independent mechanisms. Applicant submits that the same would be true with any other specific membrane proteins selected as suitable passenger peptides.

Immune and inflammatory responses

The Examiner has also suggested that unwanted immune and inflammatory responses to the viral particles and passenger peptide may contribute to the alleged lack of predictability.

Applicant has shown in previous remarks that many studies have been carried out using viruses of the virus families postulated and that these have not shown adverse immune responses to the viral particles and the passenger peptide would be selected by the skilled artisan specifically to minimize potential adverse immune responses. Any foreign protein has the potential to illicit an immune response, but any potential immunogenicity of the viral particles is believed not to be relevant to enablement or written description issues or relevant to the patentability of the current invention.

It is noted, however, that the previously cited Cronin et al (2005) has again been cited as evidence that cell-derived components are concentrated on the surface of viral particles leading to potential immune responses. It will be appreciated

that the potential targeting peptides by their nature would be cellular products and should have low immunogenicity. Indeed, any potential immunogenicity may well be exploited for the production of vaccines and is therefore not necessarily undesirable. The material presented above in relation to the mechanism of incorporation of the passenger peptide into the budding viral particle will also address some immune response concerns.

The Examiner considers that the immunogenicity of the virus will depend not only on the passenger peptide but on the expression levels of said peptide, the viral particle and on the packaging cell line used. This conclusion is not understood and is not considered a relevant factor. It seems clear that the outcome with any given passenger peptide would be considered by a person of skill to be predictable and the immunogenicity of the resulting particles would be low.

The Examiner also has raised another immune response concern having to do with the nature of the passenger peptide's association with the viral particle (it is attached to the membrane rather than covalently linked to the viral envelope protein). Because of this, it is speculated that it may dissociate from the viral particle and cause an unwanted immune response. The basis of this notion is unclear and no evidence is presented to support the allegation that the passenger peptide may spontaneously dissociate from the viral particle membrane.

The passenger peptide is necessarily a membrane associate protein (or else the invention would not work). Such proteins do not simply "fall" out of membranes. Indeed, their hydrophobic nature would make them insoluble and exceedingly unlikely to dissociate from the membrane even in the event of the destruction of the viral particle.

Viral envelope is not modified

Further, the Examiner has cited Gritsun et al (1995) as evidence that the envelope proteins of viruses are variable and that this affects the antigenicity of viral particles. Also cited is Hayasaka et al (2004), which demonstrates that mutations in the envelope protein can affect viral infectivity. These citations are intended to buttress the Examiner's position that the outcome with any given passenger peptide is not predictable.

Both Gritsun and Hayasaka relate to flaviviruses and it is clear that flaviviruses are of little relevance to the current invention. Flaviviruses cannot be used as long term gene transfer vehicles because they are RNA viruses. Moreover these references are concerned with variability in the envelope proteins of these viruses affecting their properties. The current specification does not discuss envelopes (as the inventors do not modify them) other than to specify that the envelope used needs to be incapable of binding to the target cells. It would be clear to the skilled person that micro heterogeneity in the envelopes of these viruses is not relevant.

The current invention is concerned with co-expressing passenger peptides for incorporation into the budding viral particle. Discussion of mutations in viral envelope proteins appears entirely irrelevant. The passenger peptide would be selected by the skilled person for its binding capacity when associated with the plasma membrane. Applicant believes that there is absolutely no reason to consider that such selected passenger peptides would re-combine or mutate in vitro in the packaging cell.

Further evidence to support applicant's position that the current invention is transferable to other membrane proteins and targets may be found in Yang et al (2006) Targeting lentiviral vectors to specific cell types in vivo. PNAS USA 103(31): 11479-11484 (attached as Exhibit E). The authors show that a strategy similar to that used in the current invention may be used to target lentivirus particles to B cells using antiCD20 incorporated on the surface of the viral particle.

Rejections under 35 USC § 102

The Examiner has maintained the previous rejection under 35 USC § 102(b) that the current application lacks novelty over Soong et al (2000). This rejection is respectfully traversed.

As previously noted, Soong et al did not teach the incorporation into the packaging cell membrane of a peptide that is foreign to the virus itself. Therefore the claim limitation

that the passenger peptide is not derived from the packaging cell or the virus should have overcome this objection.

As mentioned above in relation to the origin of the passenger peptide, the Examiner has again expressed some confusion as to the precise meaning of the previous amendments. The Examiner has argued that due to the alleged inconsistency, in the language of claim 43 the passenger peptide may be considered to be any peptide.

It is believed that the explanation previously presented with regard to the clarity of the claims also meets this rejection.

Additionally, Soong et al relates to altering viral tropism through modification of envelope proteins. The method in Soong could not target the SCF receptor (c-kit) as the viral envelope cannot bind c-kit. The Current invention uses a passenger peptide that is not derived from the viral particle and is not derived from the packaging cell. There is no teaching in Soong of the use of such proteins to target viral particles.

Thus, Soong et al does not disclose all the claimed elements and limitations and cannot properly support a rejection under 35 USC § 102(b). Accordingly, the Examiner is asked to reconsider and withdraw this rejection.

Rejections under 35 USC § 103

Claims 43, 48, 50 and 51 remain rejected under 35 USC § 103(a) as being unpatentable over Soong et al, taken with

Dropulic et al (USPN 6,114,141), maintaining a previous rejection. This rejection is also respectfully traversed.

The Examiner also maintains that the teaching of Soong et al provides virus particles with altered tropisms that can be used independently based on the characteristics of the intended target cells. Therefore, despite evidence presented to the contrary, the Examiner still considers that the products of in vitro evolution that are selected in Soong et al reads on the specifically designed products of the current invention. This is not the case as one cannot target a specific cellular receptor with, and direct the outcome of, the 'molecular breeding' process.

With regard to the combination with Dropulic et al, the Examiner has re-iterated the previous objection. These mainly relate to specific embodiments of the current invention, such as the bioactive molecules that may be expressed and incorporated into the virus particle.

For reasons of record, taken together with the above remarks, applicant believes that once the language of the claims is clearly understood, patentable differences are appreciated.

As previously stated, with respect to this rejection, claim 43 has been amended to specify that the passenger peptide is incorporated into the packaging cell membrane. Furthermore, as discussed above, Soong does not describe the passenger peptide as being heterologous. In addition, it is clear that Dropulic does

not describe the expression of such heterologous proteins so as to be incorporated into the viral particle via the envelope formed by budding.

Given the diversity, there is no reason or motivation to combine the teachings of these two documents as they do not complement each other. Soong is directed specifically to evolution of viral genes – there is no indication that a skilled person may want to add new heterologous genes – hence there is no incentive to combine these two documents.

Furthermore, the method of Soong is not readily adapted for dealing with different viruses or proteins. Soong requires a recombination process of evolution to be conducted each time a new final product is required. The current method allows different viruses to be replicated in the same packaging cell line in order to get different viruses with the same passenger peptide in the envelope.

Additionally, it is of interest that the inventor has also advised us that the use of recombination in gene therapy vectors is not viable ("completely taboo" being his exact words) on the basis that you do not want your vectors recombining. He also informed us that gene therapy vectors specifically have recombining sequences removed from their genomes.

Accordingly, there is no reason why the skilled person would adapt Soong to form a gene therapy vector. Reconsideration and withdrawal of this rejection is respectfully requested.

It is further noted that claims 43, 48, 52 and 53 have been newly rejected under 35 USC § 103(a) as being unpatentable over Soong et al taken with Guber et al (USPN 5,691,177). This rejection is also respectfully traversed.

In this regard, it is noted that Guber '177 is quite similar to Dropulic, the only difference being that the references disclose different cytotoxic agents. This being the case, applicant remains convinced that the remarks pertinent to the combination of Soong et al and Dropulic apply equally to the combination of Soong et al and Guber et al; and for these reasons, it is believed that the present claims patentably distinguish over the combination of Soong and Guber, as well.

The rejection of claims 43 and 47 under 35 USC § 103(a) as being unpatentable over Soong et al, taken with Yajima et al (Retroviral vector targeting human cells via c-Kit-stem cell factor interaction. *Hum Gene Ther.* 9(6): 779-87, 1998) has also been maintained. This rejection is again respectfully traversed.

Soong et al is believed to have been adequately distinguished, particularly in view of the explanatory remarks having to do with clarity of claim language and novelty. Yajima, as previously stated, does not describe enveloped viruses containing heterologous peptides derived from packaging cells at all, but describes a chimeric protein to a non-chimera and because of these differences, it is believed that one skilled in the art would not be led to combine these documents and, even if

they were combined, such a combination would not remove an inventive step from the present claims.

In view of the above amendments, taken together with the explanatory remarks herein, the Examiner is respectfully requested to reconsider and withdraw the present rejections and it is further requested that the claims thereafter be allowed.

Should minor issues remain which, in the opinion of the Examiner, could be resolved by telephone interview, the Examiner is invited to contact the undersigned attorney at his convenience to discuss and hopefully resolve same.

Respectfully submitted,

NIKOLAI & MERSEREAU, P.A.

A handwritten signature in black ink, appearing to read "C. G. Mersereau", written in a cursive style.

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CERTIFICATE OF MAILING

I hereby certify that the foregoing Amendment in response to the Non-Final Official Action of November 19, 2007, together with a Exhibits A-E, a Petition for a Three-Month Extension of Time, a Transmittal Letter, and a check in the amount of \$1050.00 in application Serial No. 10/520,745, filed on August 22, 2005, of Colin M. Casimir, entitled "METHODS OF MAKING VIRAL PARTICLES HAVING A MODIFIED CELL BINDING ACTIVITY AND USES THEREOF" are being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to: Commissioner for Patents, P.O. BOX 1450, Alexandria, VA 22313-1450, postage prepaid, on May 16, 2008.

Barbara L. Davis
On Behalf of C. G. Mersereau
Attorney for Applicant(s)

Date of Signature: May 16, 2008

Minimal exclusion of plasma membrane proteins during retroviral envelope formation

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The retrovirus forms its envelope by budding at the plasma membrane (PM). This process is primarily driven by its cytoplasmic core-precursor protein, Gag, as shown by the efficient formation of virus-like Gag particles in the absence of its envelope protein, Env. Most interestingly, several studies have demonstrated incorporation of various PM proteins into retrovirus, but the underlying mechanism of this phenomenon has remained elusive. We have purified Moloney murine leukemia virus Gag particles by sedimentation in an iodixanol gradient and donor PMs by flotation in a sucrose gradient and compared their protein compositions at equal lipid basis. We found that most PM proteins are present at similar density in both membranes. The inclusion of PM proteins was unaffected by incorporation of Env protein into the envelope of the Gag particles and whether these were produced at high or low level in the cells. These findings indicate that most PM proteins become incorporated into the retrovirus envelope without significant sorting. This feature of retrovirus assembly should be considered when studying retrovirus functions and developing retrovirus vectors.

According to a prevailing model, virus-specific membrane proteins are incorporated into the viral envelope by means of specific interactions with the viral core, whereas host membrane proteins, lacking possibilities to undergo such interactions, will be excluded (1). Although, experimental results support the validity of this model for some viruses—e.g., the alphaviruses (2)—it is not applicable to others—e.g., the retroviruses. In particular, several studies with HIV-1 suggest that many plasma membrane (PM) proteins of the host become incorporated into the viral envelope. These proteins include cell adhesion molecules such as CD44, LFA-1, and ICAM-1 and the antigen presenters HLA-I and II (reviewed in ref. 3). Similarly, studies with several different retroviruses show that they can be pseudotyped with envelope proteins of nonrelated viruses if the latter are expressed at the PM of the host cell (reviewed in refs. 4 and 5). These phenomenon may be related to the fact that retrovirus budding is not, like that of alphavirus, dependent on core-envelope protein (Env) interactions but depend on interactions of core proteins alone (reviewed in ref. 5). Thus, expression of the *gag* gene—i.e., the gene encoding the internal core protein (the Gag precursor)—in the absence of other viral genes results in formation of retrovirus-like Gag particles (reviewed in ref. 6). This Env-independent budding might favor host protein incorporation into the retrovirus envelope. However, the exact mechanism for the incorporation is still unclear. In particular, it is not known whether only certain or most PM proteins are incorporated into the retrovirus envelope and whether that incorporation occurs passively. To characterize this process it is necessary to compare the densities of PM proteins in the donor PM of the host cell and in the envelope of the retrovirus. Here we present such a study with Moloney murine leukemia virus (Mo-MuLV) Gag particles.

Materials and Methods

Cell Culture. BHK-21 baby hamster kidney cells were grown as described (7). MOV-3 mouse fibroblast cells (NIH 3T3 cells

transformed with wild-type Mo-MuLV genome) were obtained from G. Schmidt (GSF-National Research Center for Environment and Health, Neuherberg, Germany) and grown as NIH 3T3 cells as described (8).

Virus, Vectors, and Infection. Semliki Forest virus (SFV) stocks were produced in BHK-21 cells transfected with RNA transcribed *in vitro* from plasmid pSP6-SFV4 (9). The SFV vector RNAs were transcribed *in vitro* from plasmids: pSFV-C/Pr65^{gag}, which contains a SFV *capsid*–Mo-MuLV *gag* fusion gene; pSFV-1/Pr65^{gag}, which contains the Mo-MuLV *gag* gene; pSFV-1/Pr65^{gag}+Pr80^{env}, which contains the Mo-MuLV *gag* and *env* genes in two separate transcription units; and pSFV-C/NP, which contains a SFV *capsid*–influenza virus A/PR/8 *nucleo-protein* (NP) fusion gene (10, 11). Infectious SFV vectors were produced by cotransfection of cells with vector RNA and helper 1 RNA as described (12). The titers of vector stocks were determined by indirect immunofluorescence using anti-Pr65^{gag} or -NP antibodies (7, 11). For infection, nearly confluent BHK-21 cells were incubated with SFV or SFV vectors (multiplicity of infection = 5–10) for 1 h at 37°C as described (7).

Metabolic Labeling. Labeling with [³⁵S]methionine. Cells were seeded in phosphate-free Dulbecco's modified Eagle's medium supplemented with L-arginine, sodium cystine, D-glucose, L-glutamine, *D*-inositol, L-leucine, and L-methionine, as recommended by the manufacturer (GIBCO/BRL/Life Technologies), and further with 5% FCS, 20 mM Hepes, and 1/10 of the regular concentration of sodium phosphate (low-phosphate medium). After 24 h, the medium was changed to a similar one, but with only 1/10 of the regular concentration of L-methionine (low-phosphate, low-methionine medium) and supplemented with 100 μ Ci/ml [³⁵S]methionine (Amersham; 1 μ Ci = 37 kBq) ([³⁵S]methionine labeling medium). The cells were labeled for 15 h and then infected with SFV or SFV vectors. After infection, labeling was continued for 3.5 or 5.5 h in fresh [³⁵S]methionine labeling medium. Particles were collected for 30 or 60 min by incubation in new [³⁵S]methionine labeling medium, the last 15 min in excess of unlabeled methionine (300 μ g/ml).

Labeling with [³²P]orthophosphate. Cells were seeded in low-phosphate medium supplemented with 25 μ Ci/ml [³²P]orthophosphate (Amersham). After 24 h, the medium was changed to low-phosphate, low-methionine medium supplemented with 25 μ Ci/ml [³²P]orthophosphate ([³²P]orthophosphate labeling medium) and labeling was continued for 15 h. The

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Abbreviations: EM, electron microscopy; Mo-MuLV, Moloney murine leukemia virus; PM, plasma membrane; PL, phospholipids; SFV, Semliki Forest virus.

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cells were infected with SFV vectors for 1 h, and the labeling was continued in fresh [32 P]orthophosphate labeling medium for 3.5 or 5.5 h. Particles were collected for different periods in low-phosphate, low-methionine medium without further labeling.

Labeling with [3 H]uridine. Cells were grown in low-phosphate medium for 40 h, infected with SFV-C/Pr65^{gag} vectors for 1 h, and incubated in low-phosphate, low-methionine medium supplemented with 12.5 μ Ci/ml [3 H]uridine for 5 h. Finally, particles were collected in low-phosphate, low-methionine medium containing 10 μ Ci/ml [3 H]uridine for 1 h.

Isolation of Gag Particles and SFV. Media from [32 P]orthophosphate- and [35 S]methionine-labeled cells, respectively, were mixed with medium containing unlabeled carrier Gag particles (approximately 3–5 μ g) and clarified by low-speed centrifugation. The particles were isolated from the supernatant by either of two methods. (i) The medium was applied on top of 3 ml of 10% (wt/wt) sucrose in 50 mM Tris-HCl, pH 7.4/100 mM NaCl/0.5 mM EDTA (TNE) in a Beckman SW41 tube, and particles were pelleted by centrifugation for 1.5 h at 35,000 rpm and 4°C. (ii) Medium was applied on top of a 5–20% (5–30% for SFV) iodixanol gradient (wt/vol) (Optiprep, Nycomed Pharma, Oslo) in a Beckman SW41 rotor, and particles were sedimented for 1.5 h at 36,000 rpm and 4°C. The fractions (700 μ l) were diluted 2-fold with TNE, and particles were pelleted by centrifugation in a Beckman JA18.1 rotor for 1.5 h at 17,000 rpm and 4°C. Alternatively, particle-containing fractions were identified by scintillation counting, pooled, and diluted 5-fold with TNE, and particles were pelleted by centrifugation in a Beckman SW41 rotor for 1.5 h at 35,000 rpm and 4°C.

Electron Microscopy (EM). EM analyses of negatively stained (2% uranyl acetate) particles and of ultrathin sections of pelleted Pr65^{gag}-enriched PMs were done as described (7). Cryosections of MOV-3 cells and infected BHK-21 cells and subsequent labeling with biotinylated concanavalin A (Con A)/mouse anti-biotin monoclonal antibody/rabbit anti-mouse IgG/10-nm protein A-gold conjugate was performed as described (13). The biotinylated Con A and the corresponding antibody were from Sigma. The number of gold particles labeling the PM and the viral membrane was systematically sampled. The length of the membrane profile was estimated by intersection counting (14). The formula used was Q/d , where Q is the number of gold particles, I is the number of intersections, and d is the distance between the test lines (1.13 μ m).

Other Methods. Homogenization of cells and isolation of Pr65^{gag}-containing PMs by flotation in a sucrose step gradient was done as described (7). Peak fractions, identified by scintillation counting, were pooled, diluted in 10 mM Tris-HCl, pH 7.4, and pelleted by centrifugation in a Beckman SW41 rotor for 1 h at 35,000 rpm at 4°C. Chloroform/methanol extraction, protein analyses by 6–15% gradient SDS/PAGE, quantification of radioactivity in protein bands, and Triton X-114 extraction of membrane proteins were done as described (7, 15, 16).

Results

Purification of Gag Particles. We used the SFV-C/Pr65^{gag} vector for expression of the gag gene of Mo-MuLV. This is an efficient RNA vector that takes over most of the translational activity of the cell. Therefore, to follow host proteins during budding of Gag particles we labeled cells with [35 S]methionine for 15 h before vector infection and continued labeling until particles were collected. This protocol ensured steady-state labeling of both host- and vector-specific proteins. Fig. 1 *Top* shows a protein analysis of all particles released from SFV-C/Pr65^{gag}-vector infected cells, separated in an iodixanol gradient. The most intensively labeled protein in the gradient is Pr65^{gag}. Its

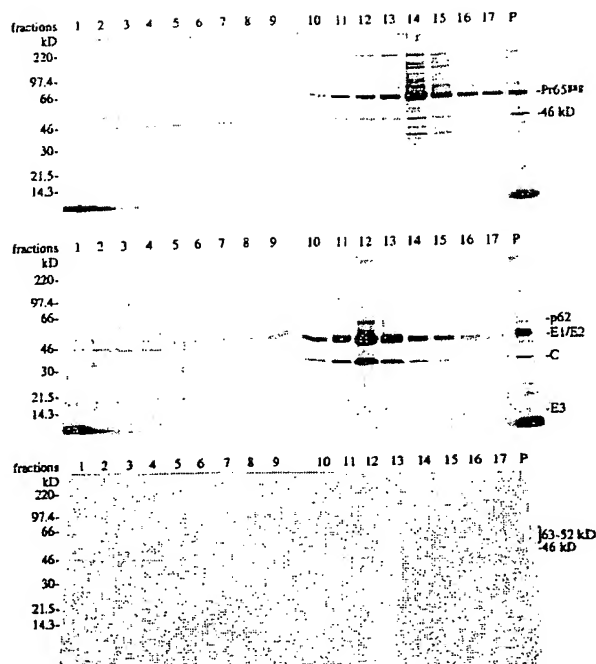


Fig. 1. Purification of Gag particles. (*Top*) Cells (4×10^6) were infected with SFV-C/Pr65^{gag} vectors and labeled with [35 S]methionine both before and after infection. Released particles in medium were collected between 5.5 and 6.0 h after infection and analyzed by sedimentation on a 5–20% iodixanol gradient. Particles were recovered from each fraction by pelleting and analyzed by SDS/PAGE. Autoradiographies of the gels are shown. Major proteins are indicated. P, pellet in gradient. (*Middle*) Cells were infected with SFV. Labeling of cells and particle analysis were as described above. Note that the iodixanol gradient was in this case 5–30%. (*Bottom*) Cells were infected with SFV-C/NP vectors. Labeling of cells and analysis of medium were as described for *Top*.

highest concentration is found in fraction 14. Of the total Pr65^{gag}, 71% is found in fractions 13–15. Most interestingly, there are several additional proteins accumulating in the same fractions. The second-most-abundant protein in the gradient is a 46-kDa protein. It is seen almost across the entire gradient with a predominance in its upper and middle parts. Furthermore, there are abundant 63- to 52-kDa proteins, which are seen in the top fractions of the gradient. This analysis suggests that several different kinds of particles have been released from the cells. Fractions 13–15, with the bulk of Pr65^{gag}, seem to contain one major class of Gag particles. Fractions 8–10, where the ratio of the 46-kDa protein to Pr65^{gag} is increased, might contain another class of Gag particles. Finally, the particles in the top fractions with the 63- to 52-kDa proteins seem to represent host-derived material. This interpretation was supported by morphological analyses using EM. The total extracellular particle preparation showed many particles that were heterogeneous in size (diameter 40–370 nm) (Fig. 2 *Left*). In contrast, the particles in the pooled fractions 13–15 showed mostly spherical particles in the size range of the Mo-MuLV virion (diameter 80–130 nm) (Fig. 2 *Right*). These results suggest that the iodixanol gradient can be used for separation of retrovirus-like Gag particles from many other particles that are also released from the SFV-C/Pr65^{gag}-infected cells.

Host-Specific Proteins Are Incorporated into Gag Particles. The additional proteins that cofractionated with the Pr65^{gag} in retro-



Fig. 2. EM analyses of released particles. Particles were produced as described in the legend to Fig. 1. (Left) Particles recovered from medium by direct pelleting through a sucrose cushion. (Right) Particles from fractions 13–15 of the iodixanol gradient in Fig. 1. (Bar = 200 nm.)

virus-like Gag particles could correspond to Pr65^{gag}-related oligomerization/degradation products, host-specific proteins of a contaminating particle population, or host-specific proteins incorporated into the Gag particles. The first possibility was tested in an experiment where we compared the protein profile of Gag particles produced in cells labeled with [³⁵S]methionine, before and after vector infection, to that of particles obtained from cells labeled only before infection. In the former case both SFV-C/Pr65^{gag}-vector and host-specific proteins should be labeled and in the latter case only host-specific ones. The results are shown in Fig. 3. The Pr65^{gag} was a prominent band in particles isolated from cells labeled both before and after vector infection (lane 1), whereas it was absent from the particles isolated from cells labeled only before infection (lane 2). Two other bands were also missing from the latter particles, namely one 33-kDa and one 38-kDa protein. The 33-kDa protein fits the size of the SFV C protein which was also produced by the vector. The 38-kDa protein could be Pr65^{gag}-derived or, alternatively, a host protein induced by vector infection. We conclude that other proteins in the Gag particles are host-specific.

The question whether the host proteins are constituents of a contaminating particle population or incorporated into the Gag particles was studied by analyzing particles released from cells that were infected with either wild-type SFV or another SFV vector, SFV-C/NP, which carries the NP gene of influenza virus A. The SFV particles are known to be virtually free from

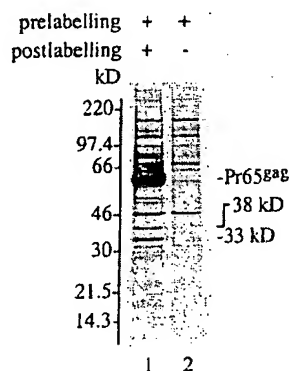


Fig. 3. Host-specific proteins cofractionate with Gag particles. Two cultures were infected with SFV-C/Pr65^{gag} vectors in parallel. One was labeled both before and after infection and the other one only before infection. The retrovirus-like Gag particles, collected 5.5–6.0 h after infection, were purified as described in the legend of Fig. 1 and analyzed by SDS/PAGE. Vector-specific proteins are indicated.

host-specific proteins, and influenza NP protein (56 kDa) cannot bud when expressed alone, but accumulates in the cell nucleus (2, 11). A sedimentation analysis of particles released from SFV-infected cells is shown in Fig. 1 *Middle*. The SFV particles, which contain the C protein, the almost comigrating spike subunits E1 and E2, and the small E3 protein as well as a small amount of the E2 and E3 precursor protein p62, peak in fractions 11–13. It is evident that there are no host-specific proteins that follow the SFV particles in the gradient. A similar analysis of the medium of SFV-C/NP-infected cells shows complete absence of protein-containing particles in the lower part of the gradient. However, both SFV- and SFV-C/NP-infected cells released slowly migrating particles with protein profiles similar to the corresponding particles from SFV-C/Pr65^{gag}-infected cells. These results show that SFV or SFV-vector-infected cells do not produce particles that sediment like Gag (or SFV) particles. Consequently, the host proteins cofractionating with the Gag particles must be constituents of these rather than contaminating particles.

Isolation of Donor PMs for Gag Particles. We have earlier described a microsome flotation procedure for isolation of a Pr65^{gag}-enriched (sub)fraction of the PM, which might be used for Gag-particle formation (7). This possibility was confirmed by a morphological characterization of these membranes by EM. This technique showed vesicular structures, many of which contained budding profiles of Gag particles (Fig. 4). These budding profiles were easy to identify by their size (100–150 nm) and characteristic multilayered surface structure.

Quantification of Phospholipids (PLs). We chose to measure surface areas of Gag-particle envelopes and PMs on the basis of their PL content. For this purpose the PLs of BHK-21 cells were steady-state labeled with [³²P]orthophosphate, and the cells were then used for production of ³²P-labeled Gag particles and isolation of ³²P-labeled PMs. The preparations were solubilized in an excess of hot SDS, and labeled material was separated by SDS/PAGE (20%). Both PM and Gag-particle preparations (Fig. 5, lanes 1 and 2) give one broad heavily labeled band in the separating gel, a faint band in the gel front, two or three very faint bands migrating slower than the broad band, and bands at the top of the separating and stacking gels. Control analysis using radioactively labeled orthophosphate (lane 3), PLs (lane 4), and RNA (lanes 5 and 6) showed that the material in the front was free orthophosphate, the broad band was PLs in SDS micelles (18 kDa) (17), and the material at the top of the separating and

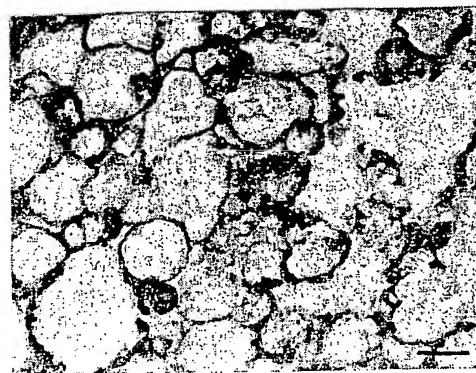


Fig. 4. EM analysis of Pr65^{gag}-enriched PMs. Cells were infected with SFV-C/Pr65^{gag} vectors and homogenized, and microsomes were separated by flotation in a sucrose step gradient. The figure shows a section of Pr65^{gag}-enriched PMs. (Bar = 200 nm.)

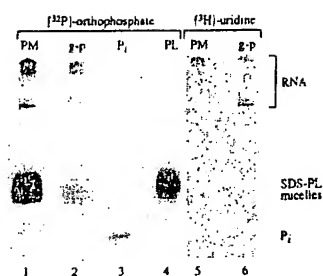


Fig. 5. Separation of PLs in PM microsomes and Gag particles by SDS/PAGE. Shown are SDS/PAGE analyses of ^{32}P -labeled PMs (lane 1) and Gag particles (g-p) (lane 2) isolated 5.0–6.0 h after infection, ^{32}P -labeled PMs (lane 3), and ^{32}P -labeled PLs, extracted by chloroform/methanol from ^{32}P -labeled PMs (lane 4). Also shown are PM and Gag particles produced in ^3H -uridine-labeled cells (lanes 5 and 6).

stacking gels was RNA. Any ^{32}P -labeled proteins were expected to migrate slower than the PLs and might correspond to the two or three very faint bands in the upper part of the separating gel. Thus, this simple procedure allowed accurate quantitation of PL content in PM and Gag-particle preparations.

Sorting of PM Proteins During Budding of Gag Particles. The behavior of the PM proteins during budding of Gag particles was studied as follows: Gag particles were produced in two cultures under identical conditions. One was labeled with [^{35}S]methionine (both before and after infection with SFV-C/Pr65^{gag} vectors) for isolation of Gag particles and PMs with labeled proteins, and the other one with [^{32}P]orthophosphate for isolation of Gag particles and PMs with labeled PLs. The ^{32}P -labeled PM and Gag-particle preparations were subjected to SDS/PAGE for quantification of total PLs, and the values obtained were used to normalize the membrane contents of the ^{35}S -labeled PM and Gag-particle preparations to each other. The labeled proteins were then analyzed by SDS/PAGE. As we were concerned about the increased accumulation of Pr65^{gag} that occurs with time in vector-infected cells, we performed all our experiments before 6 h after infection—i.e., 6–8 h before appearance of visible cytopathic effects in vector-infected cells. Furthermore, we used two different collection times for particles: one between 3.5 and 4.0 h after infection, when the Pr65^{gag} synthesis was still increasing in the cell, and another between 5.5 and 6.0 h after infection, when full gag gene expression had been achieved. Single, rather than double, labeling of cells with [^{32}P]orthophosphate and [^{35}S]methionine was used in these experiments because the intensively labeled PLs interfered with the weaker ^{35}S label in the proteins. A drawback of the separate labeling protocol was that the membrane equalization became dependent on the reproducibility of yields of PMs and Gag particles in the parallel purifications. Separate tests showed that the variability of yields was within 10% for both preparations when these were isolated at the later time point and within 25% when prepared at the early time point.

An examination of the protein profiles of Gag particles collected at the early time period and of particles collected at the later time showed that the protein compositions are virtually identical (Fig. 6, lanes 2 and 4). This result indicated that incorporation of host proteins into Gag particles was not significantly influenced by the increased accumulation of Pr65^{gag} in vector-infected cells. As steady-state labeling conditions were used in the experiment, it was possible to compare the amounts of host proteins in the particles to the amount of Pr65^{gag} by measuring their ^{35}S radioactivities. Quantification showed that the radioactivity of the most intensively labeled host proteins

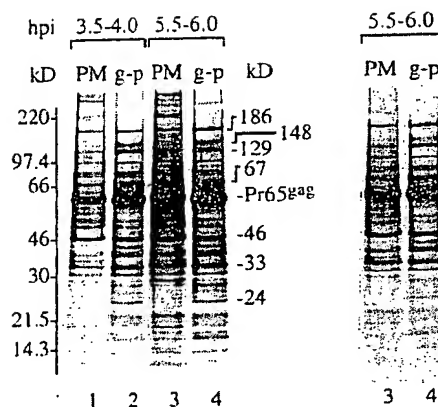


Fig. 6. Sorting of host proteins during budding of Gag particles. ^{35}S -labeled Gag particles (g-p) and PMs were adjusted to contain an equal amount of membranes and then analyzed by SDS/PAGE. Samples in lanes 1 and 2 are from a particle production between 3.5 and 4.0 h after infection and those in lanes 3 and 4 are from a production between 5.5 and 6.0 h after infection. Host proteins in Gag particles are indicated. (Right) A shorter exposure of the gel analysis of the samples from the later collection period.

each constituted 3–5% of the Pr65^{gag} radioactivity. As Pr65^{gag} contains only about 1/5 of the average frequency of methionine residues in proteins (18), this result suggests that they each constitute 0.6–1.0% of Pr65^{gag} by mass. Most surprisingly, the protein composition of the PM preparations was found to be strikingly similar to that of the Gag particles (compare lanes 1 and 2, and lanes 3 and 4). This finding suggested that the majority of the PM proteins were included in the Gag particles. A closer examination revealed that all proteins of the Gag particles were present in the PM and hence must be considered as PM proteins. The PM preparations contained, in addition, some proteins that were absent from Gag particles, or alternatively, present in very low amounts. Examples of such were the proteins migrating at the top of the gel, between the 186-kDa and the 148-kDa proteins, and also between the Pr65^{gag} and the 46-kDa protein. As samples in lanes 1 and 2 and in lanes 3 and 4, respectively, had been equalized on the basis of their PL content, we could roughly assess the sorting of individual PM proteins into the envelope of the Gag particle. At the later time point (lanes 3 and 4) the majority of the shared bands appeared with equal intensities in the two samples, suggesting no sorting but passive inclusion in a nondiluting and nonconcentrating manner into the budding particles. A few, most notably the 24-kDa protein, but also Pr65^{gag} (as expected) and the 67-kDa and 148-kDa proteins, were more concentrated in the Gag particle than in the PM, suggesting that these were actively sorted into the envelope of the Gag particle. Still other proteins, already mentioned above, were excluded from the envelope of the Gag particle. At the earlier time point (lanes 1 and 2) more proteins appeared to be concentrated in the Gag particle during budding. However, this was not a constant finding. In some experiments sorting conditions similar to those found at the later time point were observed. These differences were probably due to variation in the yields of ^{35}S - and ^{32}P -labeled Gag particles. As already noted, the variation was larger for particle preparations collected at the early time than for those collected at the later time.

Most Host Proteins in Gag Particles Are Integral Membrane Proteins. To identify integral membrane proteins in Gag particles, we subjected particles to Triton X-114 extraction and subsequent phase separation. The result is shown in Fig. 7 Left. Except for

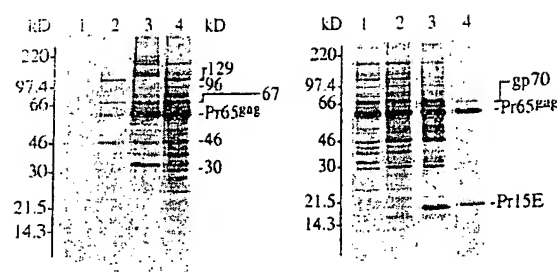


Fig. 7. Host proteins in Gag particle: Detergent binding and effect of Env coassembly. (Left) 35 S-labeled Gag particles were solubilized with Triton X-114, and protein partitioning was followed during detergent and aqueous phase separation. Unsolubilized material (lane 1) and solubilized material in the aqueous (lane 2) and detergent (lane 3) phases were analyzed by SDS/PAGE together with a sample of starting material (lane 4). Host proteins are indicated. (Right) Gag particles were produced in cells infected with SFV-1/Pr65^{gag}+Pr80^{env} and labeled both before and after infection (lane 3) or only after infection (lane 4). The particles were purified by sedimentation in an iodixanol gradient and analyzed by SDS/PAGE. Gag particles produced in cells infected with SFV-C/Pr65^{gag} (lane 1) and SFV-1/Pr65^{gag} (lane 2), respectively, were analyzed as controls.

a small amount of Pr65^{gag} (lane 1), all proteins of Gag particles were solubilized by Triton X-114. The majority of the PM-derived proteins partitioned preferentially into the detergent phase and hence were considered to be integral membrane proteins (lane 3). This was also the case with Pr65^{gag}. A few proteins partitioned preferentially into the aqueous phase (e.g., the 129-, 46-, and 30-kDa proteins) (lane 2), and some proteins distributed approximately equally between the two phases (e.g., the 96- and 67-kDa proteins). In the presence of high salt (0.5 M NaCl) the Pr65^{gag} distributed equally between the detergent and aqueous phase, whereas the other proteins partitioned as before (data not shown).

Incorporation of Env into Gag Particles Does Not Affect Host Protein Inclusion. To test the effect of Env on host protein inclusion into Gag particles we coexpressed the *gag* and *env* genes of Mo-MuLV by using an SFV-1 vector that carried separate transcription units for *gag* and *env*. As this SFV-1/Pr65^{gag}+Pr80^{env} vector drives 8-fold lower gene expression than does the SFV-C vector, we collected particles for a longer time—i.e., for 1 h between 5 and 6 h after infection (19). The SFV-1 vector was used, because high expression with the SFV-C vector caused most Pr80^{env} to form disulfide-linked aggregates (H. Andersson and H.G., unpublished results). Fig. 7 Right, lane 4, shows the 35 S-labeled proteins of Gag particles from SFV-1/Pr65^{gag}+Pr80^{env}-infected cells, labeled only after infection. Under these conditions only the Mo-MuLV-specific Pr65^{gag} and the Env subunits gp70 and Pr15E were seen. Quantification indicated that there was about one Env complex per five Pr65^{gag} molecules. When particles were produced in cells labeled both before and after vector infection, several additional proteins were seen (lane 3). The pattern of this was very similar to those of the host proteins in particles produced in cells infected with the high-level *gag*-expression vector, SFV-C/Pr65^{gag} (lane 1), and in particles produced in cells infected with a low-level *gag*-expression vector, SFV-1/Pr65^{gag} (lane 2). Note the 33-kDa protein in particles from SFV-C/Pr65^{gag}-infected cells. This is most likely vector-specific SFV C protein. We conclude that host proteins are not significantly excluded from the Gag particle by the simultaneous incorporation of the homologous Env.

Table 1. Quantitation of Con A labeling of PM with budding structures

Virus	Density, Au particles/ μ m	
	Viral membrane	PM
Mo-MuLV Gag particles	5.4 \pm 0.9	3.3 \pm 0.4
Mo-MuLV wild type	8.5 \pm 0.8	2.3 \pm 0.3
SFV	31 \pm 10.1	3.8 \pm 1.2

BHK-21 cells infected with SFV-C/Pr65^{gag}, MOV-3 cells, and BHK-21 cells infected with SFV were sectioned and labeled with Con A, and Con A was detected in EM using a combination of antibodies and protein A-gold conjugate. The number of gold particles per boundary length of the viral membrane (column 2) and PM (column 3) was estimated ($n = 3$; \pm SD).

Cryo-Immuno-EM. To extend our biochemical data to the ultrastructural level we used cryo-immuno-EM. BHK-21 cells infected with SFV or SFV-C/Pr65^{gag} vectors and NIH 3T3 cells infected with wild-type Mo-MuLV (MOV-3 cells) were cut into ultrathin sections and labeled with Con A to detect glycoproteins. The labeling density was then calculated in parts of the PM that were, or were not, involved in viral budding. This was done by quantitative estimation of the number of gold particles per boundary length (μ m). The results are presented in Table 1. The labeling density in budding-free regions of the PM is approximately the same in all infected cells (Table 1, column 3). A very similar labeling density is also found in membranes of budding Gag particles, whereas it is somewhat increased in those of budding Mo-MuLV and much higher in those of budding SFV (Table 1, column 2). Fig. 8 shows representative EM pictures of a budding Gag particle (A), an apparently released wild-type Mo-MuLV (B), and a budding SFV (C). We conclude that these results are consistent with our biochemical finding that PM proteins in general are included in the Gag particle during budding. The clear increase in Con A binding to SFV buds is most likely due to the high concentration of viral spike proteins. The less significant increase of label in budding Mo-MuLV might be due to recruitment of the glycosylated Env complexes to the particle.

Discussion

Our results show that most PM-associated proteins become passively incorporated into the envelope of Mo-MuLV Gag particles during budding. Surprisingly, coassembly of Env into the particles did not reduce their content of PM proteins. Insufficient Env incorporation appeared to be a simple explanation, but our analyses showed that the particles contained about one Env complex per five Pr65^{gag} molecules—i.e., about as much as previously reported for wild-type MuLV (20). Furthermore, we have recently demonstrated that Env becomes about 3-fold concentrated when sorted from the PM into the envelope of the Gag particle (M.H. and H.G., unpublished results). This finding suggests that passive incorporation of most PM proteins



Fig. 8. Con A labeling of budding Gag particles. Ultrathin cryosections of cells infected with SFV-C/Pr65^{gag} vectors, wild-type Mo-MuLV, and SFV were labeled with Con A, and the Con A was detected in EM using a combination of antibodies and a protein A-gold (10 nm) conjugate. (A) Con A labeling of a budding Gag particle and the adjacent PM. (B) Labeling of wild-type Mo-MuLV. (C) Labeling of budding SFV and adjacent PM. (Bar = 100 nm.)

could also occur during the formation of the wild-type Mo-MuLV. Unfortunately, this could not be studied directly because of the low production rate of wild-type particles in Mo-MuLV-infected cells.

One might argue that the host protein incorporation into Gag particles is, at least in part, a consequence of the high gene expression obtained with the SFV-C/Pr65^{gag} vector. However, we did not observe any significant effect on host protein incorporation when Gag particles were produced at early times after vector infection—i.e., when comparatively little Pr65^{gag} had been produced in the cells, or when using SFV-1/Pr65^{gag} or SFV-1/Pr65^{gag}+Pr80^{env} vectors, which produced 8- and 12-fold less Pr65^{gag}, respectively, than the SFV-C/Pr65^{gag}-vector (19) (M.H. and H.G., unpublished results). Although, this still represents about 10-fold higher Pr65^{gag} production than in wild-type Mo-MuLV-transformed cells, our results do show that the host protein incorporation is not critically dependent on the intracellular Pr65^{gag} concentration.

It should be stressed that not all PM proteins were passively incorporated into the Gag particles. A few PM proteins were found to be excluded from the particle. The reason for this exclusion remains unclear. These proteins might share some physical and topological features that excludes them from the submembranous Pr65^{gag} lattice, or they might not be part of the PM regions where Gag-particle formation takes place. A most intriguing finding was that a few host proteins were concentrated into the Gag particles. The most notable one was the 24-kDa protein. These host proteins might have functional roles in the assembly or entry process of Mo-MuLV and clearly deserve to be studied further.

The validity of our interpretations is primarily based on the purity of our Gag-particle and PM preparations and the accuracy of our PL quantifications, all of which can be criticized. We found that BHK-21 cells infected with various SFV vectors released, in general, significant amounts of host-derived vesicles, but these could be separated from the retrovirus-like Gag particles by sedimentation in an iodixanol gradient. This problem and its solution have been noted before (21–23). The PM preparation isolated by the sucrose step gradient was most likely to some extent contaminated by other membranes. However, the facts that our PM preparation carries a PM marker protein (7), is separated from endoplasmic reticulum and trans-Golgi membranes (7), and is significantly enriched in Pr65^{gag} and Gag-

particle budding profiles (this study) make us confident about its suitability as a donor membrane preparation for the Gag particles in our experiments. Our estimation of the PM and Gag-particle membrane ratio was based on PL quantification. The results were interpreted assuming similar lipid composition in the two preparations. This assumption might, however, be somewhat erroneous. The lipid composition of retroviruses has been studied before, and the results suggest that they contain relatively more sphingolipids and cholesterol than the PM of the host cell (24, 25). If this is also the case with Gag particles produced in BHK-21 cells, it means that the envelope of the Gag particles contain less labeled PLs per unit area than the host PM and that we hence overestimate the protein densities in the envelope of the Gag particles. However, it is unclear how much the intercalation of additional cholesterol molecules in between the PL molecules of a membrane actually increases the surface area of the latter (26).

Our biochemical results were corroborated by our immunocytochemical analyses at ultrastructural level. The Con A labeling resulted in similar densities of label in budding and budding-free regions of the PM. Assuming that Con A detects glycoproteins rather than glycolipids, we have interpreted our results as the presence of similar concentrations of glycoproteins in the two regions. The validity of our assumption is supported by the facts that (i) Con A binds preferentially to mannose, which is a frequent component of the sugar units of membrane glycoproteins but not glycolipids (14, 27) and (ii) we observe a much increased Con A labeling in SFV buds, structures which are known to involve spike protein clustering.

Bulk incorporation of PM proteins into the retrovirus envelope gives a natural explanation for the frequent observations of various host PM proteins (and functions) in retroviruses as well as for the pseudotyping phenomenon (3, 4). Moreover, it can explain the significant unspecific binding of retrovirus to cells (28). The realization of this process opens up new possibilities for modulation of the targeting and fusion functions of retrovirus vectors. However, it also raises concerns about vector purity and PM protein transport from producer cells to target cell surfaces.

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humidity for 12 to 14 days [1 to 2 days longer than in (15)] or until no additional flies emerged.

The current optimal procedures produce overall survivals that constitute effective and practical cryobiological preservation of the Oregon R strain. Although it remains to be determined whether they will also be effective in preserving embryos from mutant lines, the major developmental processes are completed by stage 16, and consequently we expect that most laboratory strains will tolerate these vitrification procedures as well as Oregon R does. An advantage of defining the optimum developmental time for cryopreservation in relation to the time at which the ratio of stages 14 to 15 is 1:1 is that it will automatically compensate for any strain-to-strain differences in development rate. The approach may also be applicable to the cryopreservation of embryos from other diptera like the housefly or mosquito, which have developmental rates very different from that of *Drosophila* (20).

We believe our findings have more general implications for cryobiology. The optimal developmental stages being frozen are probably the most complex systems that have been cryobiologically preserved. The embryos are highly differentiated into tissues and organs including muscle and nerve, which indicates that differentiated multicellularity is not a barrier to cryopreservation per se. The findings also represent perhaps the first case in which vitrification procedures are required to obtain survival. From the mechanistic point of view there remains the question of why *Drosophila* survival is so critically dependent on developmental stage. Older embryos may vitrify more readily than younger embryos, or possibly they tolerate the presence of ethylene glycol or small amounts of ice better than younger embryos, perhaps because the critical steps of dorsal closure and head involution are completed. The answers could be important in determining the extent to which the general strategies described prove applicable to other non-mammalian eggs and embryos, most of which have not been successfully cryobiologically preserved.

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21. Permeabilization was effected by successive exposure of embryos on 25-mm Nuclepore or Poretics polycarbonate filters to 2.5% hypochlorite to remove the chorion, a thorough water wash, isopropanol treatment, air-drying for 2 min to remove the isopropanol, and a mixture of 0.3% 1-butanol in *n*-heptane for 90 s, followed by a brief chase with pure heptane. Details are given in (7) and (15). The duration of the air-drying and the butanol/heptane steps are critical. The filters were rinsed several times in D-20 and then incubated 45 min at 24° to 25°C. The percentage of permeabilized embryos was defined as the percentage that stained red or dark pink after a 5-min exposure at 23°C to 0.1% rhodamine B in D-20. [The leftmost and rightmost points of the dashed curve are from (15).] The embryos on the filters were then exposed to 2 M ethylene glycol at room temperature (23°C) for 30 min and then to 8.5 M ethylene glycol plus 10% (w/v) polyvinyl pyrrolidone (Plasdone C-30) for 4.5 to 5.5 min at 5°C. The next step was to abruptly plunge the filters into a mixture of solid and liquid nitrogen (nitrogen slush) at approximately –205°C and then 10 to 30 s later to abruptly plunge the filters into 0.75 M sucrose in D-20 at 23°C, hold them in that solution for 2 min, and then transfer them into D-20. Details of and reasons for these various steps are given in (15).
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Cellular Proteins Bound to Immunodeficiency Viruses: Implications for Pathogenesis and Vaccines

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Cellular proteins associated with immunodeficiency viruses were identified by determination of the amino acid sequence of the proteins and peptides present in sucrose density gradient-purified human immunodeficiency virus (HIV)–1, HIV-2, and simian immunodeficiency virus (SIV). β_2 microglobulin (β_2m) and the α and β chains of human lymphocyte antigen (HLA) DR were present in virus preparations at one-fifth the concentration of Gag on a molar basis. Antisera to HLA DR, β_2m , as well as HLA class I precipitated intact viral particles, suggesting that these cellular proteins were physically associated with the surface of the virus. Antisera to class I, β_2m , and HLA DR also inhibited infection of cultured cells by both HIV-1 and SIV. The specific, selective association of these cellular proteins in a physiologically relevant manner has major implications for our understanding of the infection process and the pathogenesis of immunodeficiency viruses and should be considered in the design of vaccines.

Studies with subunit vaccines have shown that immunizations with viral envelope antigens alone are sufficient to elicit protective immunity against SIV or HIV (1). The recent observation (2) that macaques immunized with uninfected human cells were protected against challenge with SIV grown in human cells raised the possibility that immune responses to cellular antigens might

also be involved in protection. However, the putative cellular antigens that may be stimulating the protective response have not been identified, and the mechanism of protection is unclear.

To identify the specific cellular antigens associated with immunodeficiency viruses, we purified and sequenced proteins from preparations of HIV-1, HIV-2, and SIV.

Viruses were propagated in H9 cells, concentrated by sucrose density gradient ultracentrifugation, and centrifuged to pellet the virus (3). Proteins from purified virus were separated by reversed-phase high-pressure liquid chromatography (rp-HPLC) as described (4) (Fig. 1). The predominant cellular proteins found in purified HIV-1, HIV-2, and SIV were β_2 microglobulin (β_2m), human lymphocyte antigen (HLA) DR (α and β chains), actin, and ubiquitin and were identified by amino acid sequence analysis of the purified proteins. Actin and ubiquitin were found associated with all purified retroviruses examined, which included murine, feline, and nonhuman primate type C viruses; murine type B virus; primate type D virus; primate and ungulate lentiviruses; bovine leukemia virus; and human T cell lymphotropic virus type I (HTLV-I). However, analysis of HIV-1_{MN}, HIV-1_{IIIb}, HIV-2_{ISY}, SIV_{Mne}, and SIV_{Cat} (as in Fig. 1) revealed substantial amounts of β_2m and HLA DR, showing that these cellular proteins were common to many strains of primate immunodeficiency viruses. Purified proteins, including β_2m and HLA DR, were quantitated by amino acid analysis. For each virus, the molar ratio of β_2m and HLA DR to Gag protein was approximately 0.15 to 0.2. Assuming 2500 to 3000 Gag molecules per virion (3), we calculated that there are between 375 and 600 molecules of β_2m and HLA DR (α and β chains) per virion in the virus preparations. These calculations suggest that these specific cellular antigens outnumber the molecules of the envelope glycoprotein, gp120, on the virus [it has been previously estimated that there are approximately 216 molecules of gp120 per virion (5)]. We recovered more β_2m and HLA DR than gp120 during rp-HPLC purification of immunodeficiency viruses.

β_2m is a highly conserved protein that noncovalently associates with the HLA class II polymorphic proteins, termed heavy or α chain, to form the heavy (α) and light (β_2m) chain combination that is expressed on the surface of most, if not all, nucleated mammalian cells. Bovine β_2m from fetal bovine serum will exchange with β_2m complexed with the α chain on the surface of cells (6), and both human and bovine β_2m were found in virus preparations (Fig. 1). However, we

have detected only trace amounts of α chain (copurifying with actin, Fig. 1B) in fractions from the rp-HPLC-purified viruses, suggesting that the amounts of β_2m in the virus preparation may be in excess of the amounts of HLA class I α chain.

HLA class II antigens are primarily expressed on cells of the monocyte-macrophage lineage, dendritic cells, B cells, and activated T cells and consist of heterodimers encoded by distinct gene regions (HLA DR, HLA DQ, or HLA DP) of the

Fig. 1. Protein separation by rp-HPLC. Approximately 175 mg of purified HIV-1_{MN} was disrupted, and the viral proteins and peptides were separated by preparative rp-HPLC as described (5). Eluted proteins and peptides were detected by ultraviolet absorption at 206 nm. Proteins were eluted at room temperature with a gradient of increasing acetonitrile concentration (0 to 60%) (A). The temperature was raised to 50°C, and elution continued with a 0 to 100% 1-propanol gradient (B). HIV-1 proteins are designated as p2, p7, p1, p6, p17, and p24 (Gag proteins) and as gp120 and gp41 (envelope proteins) (5). Other peptides with Gag sequences are labeled as A through H. Pol-coded products were also found (in minor peaks) but are not labeled. The β_2 microglobulins from bovine and human sources are designated as Bovine β_2m and Human β_2m , respectively. Ubiquitin, HLA DR (β chain), actin, and HLA DR (α chain) are also indicated.

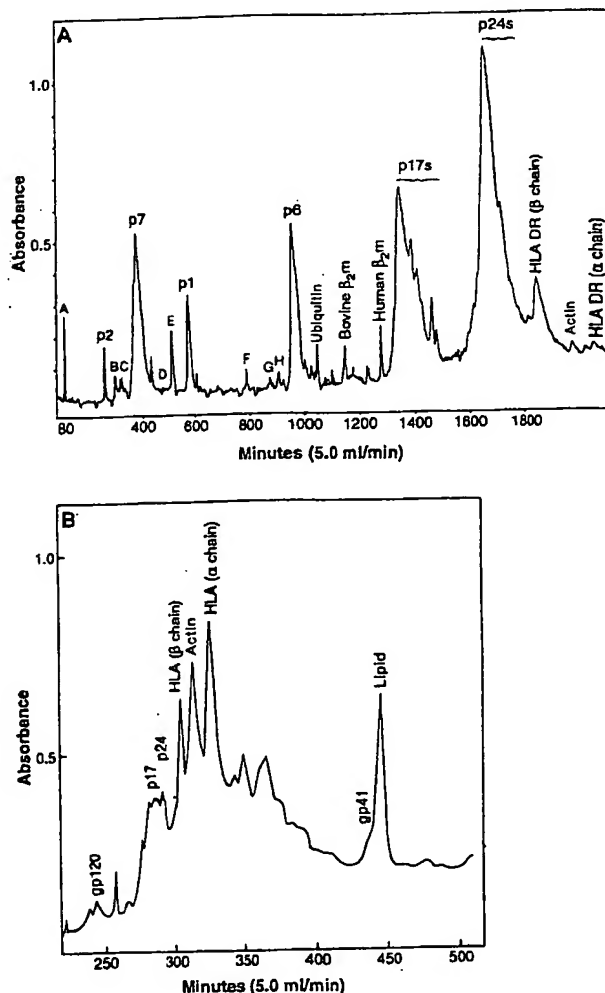
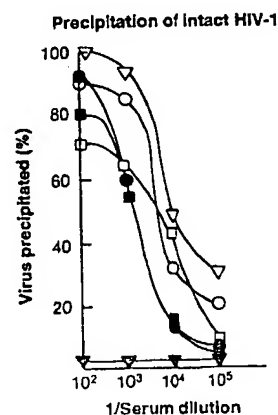


Fig. 2. Precipitation of virus by antisera to cellular proteins. Twenty microliters of each serum at the appropriate dilution was mixed with 280 μ l of phosphate-buffered saline and 100 μ l of HIV-1_{MN}. The mixture was incubated for 1 hour at 37°C and then overnight at 4°C; after the incubation, 50 μ l of 10% *Staphylococcus aureus* was added to facilitate precipitation of immune complexes. After a 30-min incubation at room temperature, pellets were prepared by centrifugation for 30 min at 3000 rpm with a Beckman J-6M centrifuge. Approximately 50 μ l of supernatant was removed, added to 950 μ l of 2% Triton X-100, and incubated for 1 hour at 37°C. We measured HIV-1 p24 in this lysate by using a DuPont HIV-1 p24 antigen capture assay, according to manufacturer's instructions. The antisera used in the assay are listed in Table 1. These include antisera to H9 cells (∇), β_2m (\circ), HLA DR (\bullet), HLA class I (\boxplus), HLA class I α chain (\square), and negative sera (actin, CD4, mouse immunoglobulin G, and bovine serum albumin) (∇).



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major histocompatibility complex. Only HLA DR was present in virus preparations, which is in agreement with the report that HLA DR but not HLA DP or HLA DQ is found on virus by fluorescence-activated cell sorter analysis (7). Infection of CEM cells with HIV-1 is known to up-regulate expression of HLA DR (8). To see if up-regulation of HLA class II on H9 cells after HIV-1 infection accounted for the preferential incorporation of HLA DR into the virus, we determined the relative concentrations of HLA DR, HLA DQ, and HLA DP on HIV-1-infected and uninfected H9 cells by means of flow cytometry as de-

scribed (9) (Table 1). Both the HIV-1-infected and uninfected H9 cells expressed HLA DR, HLA DQ, and HLA DP in the same relative concentrations, indicating that infection had not selectively altered the concentrations of the antigens on the cell surface. The presence of HLA DQ and HLA DP on the surface of HIV-1-infected H9 cells, but not in purified viruses, indicates a selective incorporation of HLA DR over HLA DP and HLA DQ.

To determine if these viral-associated cellular antigens were physically attached to HIV-1, we tested antisera specific for β_2m and HLA DR for their ability to precipitate intact virus. Antisera to uninfected H9 cells, β_2m , HLA DR, HLA class I, and HLA class I α chain efficiently precipitated intact virus (Fig. 2), indicating that cellular proteins were physically attached to the virus surface. Precipitation of intact virus with monoclonal antibodies to class I α chain (HC-10) and to conformationally dependent epitopes on HLA class I (W6/32) indicates that some of the viral-associated β_2m is complexed with α chain, forming complete HLA class I molecules on the surface of most of the virus particles. Antisera to actin, CD4, mouse immunoglobulin G, and bovine serum albumin did not precipitate the virus. Furthermore, monoclonal antibodies to HLA DR (but not to HLA DP or HLA DQ) precipitated intact HIV-1 (10).

Neutralization of HIV-1 and SIV infection by specific antisera has proven useful for identification of viral and cellular proteins involved in infection and for localization of regions of the protein that interact during the infection process. To determine if the viral-associated β_2m and HLA DR proteins were involved in virus infection, we tested antisera to β_2m and HLA DR in vitro for their ability to neutralize HIV-1 and SIV infection. Antisera to β_2m and HLA DR efficiently inhibited HIV-1 and SIV infection, as shown in Table 2. Monoclonal antibody to β_2m , B2g2-2 (11), also neutralized infection of human peripheral blood lymphocytes by HIV-1_{NDK} and HIV-1_{LAV} propagated in CEM cells (12). Because β_2m and HLA DR are expressed on the surface of cells, it was necessary to determine whether the neutralization was due to the binding of antisera to antigens on the cells or on the virus. We preincubated cells with antisera to β_2m or with a monoclonal antibody (OKT4a) that inhibits gp120-CD4 interactions by binding to a reactive site on the CD4 (13); the cells were then washed to remove antisera, and HIV-1 was added to the cells. Whereas no inhibition of HIV-1 infection was seen with anti- β_2m , and only high concentrations of anti-DR sera inhibited HIV-1 infection, preincubation with OKT4a completely prevented virus infection (14), suggesting that the β_2m and HLA

DR on the virus, and not on the cell, were the primary targets for neutralization by the respective antisera. Neutralization of HIV-1 and SIV infection by antibodies to β_2m and HLA DR is further evidence that these cellular proteins are physically associated with HIV-1 and SIV and suggests that these viral-associated cellular proteins are involved in the infection process.

We previously identified HLA DR molecules in sucrose density gradient-purified HIV-1 (15). Also, it has been shown that antisera to selected cellular antigens react with HIV-1 and SIV (16). However, we now implicate specific viral-associated cellular proteins in the infection process. The importance of understanding the infection process of immunodeficiency viruses is emphasized by differences in the pathogenic potential of HIV-1 isolates that appear to be attributable to efficiency of virus entry (17). We propose that β_2m , HLA DR, HLA class I, and gp120 on the immunodeficiency viruses interact with specific ligands on the cell (similar to binary interactions between T cells and antigen-presenting cells), allowing the formation of an "adhesion patch" of clustering molecules on the cell surface. This adhesion patch may then serve to fuse the virus and cell membranes and allow the viral core-associated genome to penetrate the cell. The presence of HLA DR on immunodeficiency viruses also has significant implications for our understanding of the pathogenesis of HIV-1 infection. The function of HLA class II molecules is to present antigens, in the form of peptides, to T cell receptors (TCR) in the initiation of cellular and humoral immune responses of the host. Inappropriate signaling after class II binding to TCR can cause apoptosis (or programmed cell death) of the TCR-bearing cell, which has been proposed as the pathological mechanism for induction of immunosuppression in AIDS (18). We propose that binding of viral-associated HLA DR to the TCR on CD4 cells may eliminate the CD4 cells by apoptotic mechanisms.

Our evidence suggests that consideration of immunodeficiency viruses should now be expanded to include cellular proteins as an integral and functioning part of the viral envelope. Whether the cellular proteins are components of the viral envelope acquired during budding or are acquired after budding by specific associations is an important mechanistic consideration that can now be addressed. In any case, it is likely that immune responses to HLA DR, HLA class I, or β_2m (or to all three cellular proteins) were involved in protection against SIV infection after immunization with uninfected human cells (2). We propose that the cellular proteins associated with immunodeficiency viruses play a cen-

Table 1. Analysis of relative concentrations of HLA antigens in HIV-1-infected and uninfected cells. Fluorescein-labeled monoclonal antibodies against determinants common to products of alleles at class I loci (HLA A, B, and C) and class II loci (HLA DR, DQ, and DP) were added at saturating concentrations to the infected and uninfected cells. The mean fluorescence was determined by flow cytometry. Isotypically matched monoclonal antibodies served as controls.

Cells	Mean fluorescence				Control
	HLA class II			HLA class I	
	DR	DP	DQ	ABC	
HIV-1-infected H9	165	94	50	68	5
Uninfected H9	164	76	43	72	5

Table 2. Neutralization of SIV and HIV-1. Neutralization assays were performed on the AA-2 CL 1 cell line as described (1). HIV-1_{IIIIB} and SIV_{mac} were grown in Hut 78 cells. Antiserum to H9 cells was prepared by immunization of a rabbit with "mock virus" prepared from uninfected H9 cells (4); anti-human β_2m was obtained from Polysciences; anti-HLA class I was monoclonal antibody (W6/32) from DAKO; and anti-HLA DR (R.DRAB1) was provided by P. Cresswell (19). Neutralization titer is expressed as the serum dilution that resulted in 50% or greater reduction in giant cell formation on the AA-2 CL 1 cells infected with SIV or HIV-1. NT, not tested.

Serum	Neutralization titer	
	SIV _{mac}	HIV-1 _{IIIIB}
Anti-H9	320	80
Anti- β_2m	704	1408
Anti-HLA DR	640	1280
Anti-HLA class I	1280	1280
SIV _{mac} -infected macaque	5632	<22
HIV-1-infected human	NT	1408
Normal rabbit	22	<22
Normal macaque	<22	NT
Normal human	22	22

mal role in infection and pathogenesis. These proteins should be considered in elucidation of the steps involved in infection, design of vaccines, preparation of experimental virus-challenge stocks, and determination of the pathological mechanisms of immunodeficiency viruses.

Protective Effects of a Live Attenuated SIV Vaccine with a Deletion in the *nef* Gene

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Vaccine protection against the human immunodeficiency virus (HIV) and the related simian immunodeficiency virus (SIV) in animal models is proving to be a difficult task. The difficulty is due in large part to the persistent, unrelenting nature of HIV and SIV infection once infection is initiated. SIV with a constructed deletion in the auxiliary gene *nef* replicates poorly in rhesus monkeys and appears to be nonpathogenic in this normally susceptible host. Rhesus monkeys vaccinated with live SIV deleted in *nef* were completely protected against challenge by intravenous inoculation of live, pathogenic SIV. Deletion of *nef* or of multiple genetic elements from HIV may provide the means for creating a safe, effective, live attenuated vaccine to protect against acquired immunodeficiency syndrome (AIDS).

There are good reasons for believing that development of an effective vaccine for AIDS will be a difficult task. Infection of humans with human immunodeficiency virus type-1 (HIV-1) and of rhesus monkeys with SIVmac is fatal most or all of the time despite an apparently strong host immune response to the infecting virus. Infected individuals maintain vigorous humoral and cellular immune responses for months or years only to succumb eventually to the virus. The ineffective nature of the natural immune response suggests that a vaccine will have to provide highly stringent protective immunity, perhaps even sterilizing immunity, in order to achieve protection. These difficulties are compounded by the large number of HIV-1 strains that are non- or minimally cross-neutralizing.

The predicted difficulty in achieving protection against HIV and SIV has been borne out to varying degrees by vaccine trials in animal models. Inactivated whole virus has protected rhesus monkeys against challenge by live, pathogenic SIV (1-4) but primarily under highly specific conditions that use human cells for the production of both vaccine antigen and challenge virus. Even formalin-fixed, uninfected human cells can provide protection under these conditions (5). Apparently, an immune response to some cellular antigen or antigens is critical for this protection. The inactivated whole virus vaccine approach has been largely unsuccessful against SIV grown in rhesus monkey lymphocytes (6, 7). Priming with vaccinia recombinants followed by boosting has protected rhesus monkeys against challenge by homologous cloned SIV (8), but little or no protection has been observed against homologous uncloned SIV (9-11). Tests of several prod-

ucts have shown only limited success in chimpanzee trials (12). What is most disappointing about these studies is that the numerous failures have occurred despite extensive efforts to maximize, in an unrealistic fashion, the likelihood of vaccine protection. The vast majority of studies have used a minimal dose of challenge virus, matched to the strain used for vaccination, at or near the peak of vaccine-induced immune response.

We investigated an approach that uses live attenuated SIV as a vaccine. Six rhesus monkeys that were infected with cloned SIVmac239 that contained a constructed deletion in the auxiliary gene *nef* have maintained extremely low virus burdens and normal CD4⁺ lymphocyte concentrations and have remained healthy for more than 3 years after experimental inoculation with the mutated virus (13, 14). Eleven of twelve rhesus monkeys infected with wild-type SIV in parallel have died over this same period. The rhesus monkeys infected with SIVmac239/*nef*-deletion have shown no clinical signs whatsoever over the entire period of observation.

Four of the rhesus monkeys infected with *nef*-deleted SIV were challenged with

Table 1. Challenge of rhesus monkeys immunized with SIV deleted in *nef*. All four rhesus monkeys received a single inoculation of SIVmac239/*nef*-deletion 2.25 years before formal challenge. Attempts to recover SIV from 10⁶ PBMCs on the day of challenge yielded negative results (dashes). The titers of neutralizing antibodies were measured on the day of challenge (1).

Rhesus monkey	SIV recovery	Antibody titer	Challenge virus
353-88	—	1:1280	SIVmac239/ <i>nef</i> -open
397-88	—	1:1280	SIVmac239/ <i>nef</i> -open
71-88	—	1:320	SIVmac251
255-88	—	1:2560	SIVmac251

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Differential Incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and Major Histocompatibility Complex Class I and II Molecules into Human Immunodeficiency Virus Type 1 Virions and Microvesicles: Implications for Viral Pathogenesis and Immune Regulation

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Human immunodeficiency virus (HIV) infection results in a functional impairment of CD4⁺ T cells long before a quantitative decline in circulating CD4⁺ T cells is evident. The mechanism(s) responsible for this functional unresponsiveness and eventual depletion of CD4⁺ T cells remains unclear. Both direct effects of cytopathic infection of CD4⁺ cells and indirect effects in which uninfected “bystander” cells are functionally compromised or killed have been implicated as contributing to the immunopathogenesis of HIV infection. Because T-cell receptor engagement of major histocompatibility complex (MHC) molecules in the absence of costimulation mediated via CD28 binding to CD80 (B7-1) or CD86 (B7-2) can lead to anergy or apoptosis, we determined whether HIV type 1 (HIV-1) virions incorporated MHC class I (MHC-I), MHC-II, CD80, or CD86. Microvesicles produced from matched uninfected cells were also evaluated. HIV infection increased MHC-II expression on T- and B-cell lines, macrophages, and peripheral blood mononuclear cells (PBMC) but did not significantly alter the expression of CD80 or CD86. HIV virions derived from all MHC-II-positive cell types incorporated high levels of MHC-II, and both virions and microvesicles preferentially incorporated CD86 compared to CD80. CD45, expressed at high levels on cells, was identified as a protein present at high levels on microvesicles but was not detected on HIV-1 virions. Virion-associated, host cell-derived molecules impacted the ability of noninfectious HIV virions to trigger death in freshly isolated PBMC. These results demonstrate the preferential incorporation or exclusion of host cell proteins by budding HIV-1 virions and suggest that host cell proteins present on HIV-1 virions may contribute to the overall pathogenesis of HIV-1 infection.

The envelope of human immunodeficiency virus type 1 (HIV-1) is comprised of host cell membrane-derived proteins and lipids incorporated into the envelope when the virion buds from an infected cell (reviewed in references 34 and 48). More than 20 different host cell-derived proteins have been identified in the HIV-1 envelope, including major histocompatibility complex class I (MHC-I) and MHC-II; the adhesion molecules CD44; LFA-1, -2, and -3; and ICAM-1 and ICAM-3 (2, 4, 21, 33). These virion-associated, host cell-derived proteins can serve as markers by which to identify the type of cell from which a virion budded (4, 6, 15). The molecular phenotype of the HIV virion envelope has been used to determine whether HIV virions produced *in vivo* budded from a macrophage (MΦ) or an activated T cell (27, 38). Incorporation of host cell-derived proteins into virions is not random or simply a function of expression level or density on the cell surface, since proteins that are highly expressed on infected cells, such as CD4, CD45, and the coreceptors CXCR4, CCR3, and CCR5, are not incorporated into virions (7, 15, 21, 25, 29).

Many cellular proteins incorporated into HIV-1 virions retain their biological function. For example, CD44 on the virion has been shown to bind hyaluronic acid (20) and CD55 (decay-accelerating factor) or CD59 present in the virion envelope can provide resistance to complement-mediated lysis (42, 43). The HIV virion envelope is enriched for HLA-DR but not DP or DQ (2, 6, 18, 45), and virion-associated MHC-II can bind and present the superantigen *Staphylococcus* enterotoxin B to resting T cells, resulting in T-cell activation (39). These observations demonstrate that virion-associated host cell proteins are functional and may play a role in HIV pathogenesis.

Normally, T cells require two signals to become fully activated. Signal one is antigen (Ag) specific and is generated by binding of the T-cell receptor (TCR) to Ag-MHC complexes on the Ag-presenting cell (APC). The second signal, a costimulatory signal, is generated by CD28 on the T cell interacting with CD80 (B7-1) or CD86 (B7-2) on an APC (reviewed in reference 19). We have previously reported that microvesicles and HIV-1 virions incorporate high levels of MHC-I and MHC-II upon budding (2, 5) and have hypothesized that virion- or microvesicle-associated MHC-I or MHC-II, with or without bound antigenic peptides, could bind to and signal through the TCR on responding T cells. It has not been previously determined whether CD80 and CD86 are incorporated into budding HIV-1 virions or microvesicles. Because TCR

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signaling in the absence of costimulation can lead to anergy or apoptosis, we examined whether microvesicles and/or HIV-1 virions incorporate CD80 or CD86 into their membranes. Here we report that HIV infection of cell lines, MΦ, and primary peripheral blood mononuclear cells (PBMC) upregulates cell surface expression of MHC-II and that virions derived from all of these cells incorporated MHC-II. CD86 was detected on virions produced from 17 of 21 sets of different virus isolates propagated on different cells, whereas CD80 was detected on virions from only 3 of the same 21 viruses produced from CD80- and CD86-expressing cells. Microvesicles were also enriched for CD86, whereas CD80 was excluded. CD45 was identified as a protein that was highly expressed on microvesicles but not on HIV-1 virions.

These data suggest that HIV has evolved to preferentially incorporate some immunoregulatory proteins, such as MHC-II and CD86, but to exclude other proteins like CD45 and CD80. The host cell molecules incorporated into virions influenced the biological effects of the virus. Noninfectious, MHC-containing HIV virions derived from the CEMX174/T1 cell line triggered cell death in resting PBMC, whereas noninfectious, MHC-negative virions derived from the matched CEMX174/T2 cell line did not. These findings suggest that HIV has evolved to preferentially incorporate certain immunoregulatory proteins into virions, potentially contributing to the ability of the virus to evade the immune system and contribute to pathogenesis.

MATERIALS AND METHODS

Cell lines. Uninfected cell lines H9 (13), CEMX174/T1, CEMX174/T2 (44), and TBLCL-CD4 (30) were cultured in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin G at 100 U/ml, and streptomycin sulfate at 100 µg/ml (complete medium). Chronically HIV-1-infected cell lines MN/H9, NL4-3/H9, NL4-3/CEMX174/T1, NL4-3/CEMX174/T2, and NL4-3/TBLCL-CD4 were also cultured in complete medium. All cell lines were split twice weekly at 3×10^5 cells/ml, were mycoplasma negative (PCR Mycoplasma Detection Kit; American Type Culture Collection, Manassas, Va.), and were cultured in complete medium.

Virus stocks and preparation of virions inactivated by Aldrichol-2 (2,2'-dithiodipyrindine). H9, CEMX174/T1, CEMX174/T2, and TBLCL-CD4 cells chronically infected with HIV-1_{NL4-3} were cultured as described previously (35). For experiments involving the induction of cell death, conformationally authentic noninfectious HIV-1 virions were prepared as previously described (1, 40). Concentrated (1,000×) virus preparations were produced by sucrose density gradient banding in a continuous-flow centrifuge (1, 5, 40). For the virion precipitation experiments, different HIV-1 isolates were examined, including patient isolates P108-436, P2-285, P419, and P115 derived from *ex vivo* expansion of primary PBMC (a generous gift from Antonio Valentin, National Cancer Institute [NCI] at Frederick). Clade B, R5 patient isolates HIV-1_{91US054}, HIV-1_{92US727}, and HIV-1_{92US657} (49), grown in PBMC activated with phytohemagglutinin (PHA) plus interleukin-2 (IL 2; 10 U/ml; Hoffman-La Roche, Nutley, N.J.), were acquired from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program. HIV-1_{SF162} (R5) (10), HIV-1_{89.6} (X4 and R5 dual tropic) (12), and HIV-1_{NL4-3} (X4) were also grown in PBMC activated with PHA-plus-IL-2 (47). HIV-1_{AD8-M} (17) and HIV-1_{RA-L} (16) were produced from primary monocyte-derived MΦ (MDM) cultures (see below). Microvesicles, used as a control reagent, were isolated from supernatants of uninfected cell cultures in a manner identical to that used for virus preparation from infected cells (5). All virus and microvesicle stocks were stored at -70°C or in vapor phase liquid nitrogen until use.

Isolation and culture of PBMC and MΦ. PBMC were isolated by density centrifugation (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden) from citrate-anticoagulated peripheral blood obtained from healthy, HIV-1-seronegative donors at the NCI at Frederick. PBMC were cultured in AIM-V medium (Gibco, Gaithersburg, Md.) with 2% human AB serum (Sigma, St. Louis, Mo.). Elutriated monocytes from HIV-negative donor leukopacs were grown at 2×10^6 cells per well on ultralow-attachment six-well Costar plates in RPMI 1640 medium

(Biosource International, Camarillo, Calif.) supplemented with penicillin, streptomycin, gentamicin, amphotericin B, L-glutamine (Quality Biological, Gaithersburg, Md.), HEPES buffer (Sigma), and 10% fetal bovine serum (Biosource International). The monocytes were incubated at 37°C under 7% CO₂ and 90% humidity for 7 days to generate MDMs. MDMs were infected with 10 50% tissue culture-infective doses of either HIV-1_{RA-L} or HIV-1_{ADA} for 2 h, washed with phosphate-buffered saline (PBS; Biosource International) to remove free virus, and refed with culture medium. The infected MDMs were incubated for an additional 18 days with medium changes every 5 days. MDMs were stained on day 18 for intracellular HIV-1 core antigen using the KC57 monoclonal antibody (MAb; Beckman-Coulter, Miami, Fla.) and determined to be greater than 80% infected (data not shown). Culture supernatants were found to be positive for HIV-1 p24 by enzyme-linked immunosorbent assay (Beckman-Coulter) at 14 days postinfection. At day 18 postinfection, culture supernatants were harvested and the MDMs were recovered by centrifugation for flow cytometric analysis.

Cell counts and viability. Total cell numbers and viability were determined by trypan blue analysis. Cells were counted on a hemacytometer in triplicate, and the percentage of dead cells was determined by the formula $[\text{dead}/(\text{live} + \text{dead})] \times 100$. Error bars represent 1 standard deviation of the mean. *P* values were calculated by using a one-tailed, equal-variance Student *t* test of experimental measurements versus a PBS control. Statistical analysis was performed with Microsoft Excel (Microsoft, Redmond, Wash.).

Flow cytometry. Immunofluorescent staining of PBMC and MDMs (3×10^5 per condition) was performed at 4°C for 30 min by using isotype immunoglobulin G1 (IgG1) (X40), IgG2a (X39), and V4 (non-gp120-interacting domain on CD4) and HLA-DR (L243) MAbs from Becton Dickinson Immunocytometry Systems (San Jose, Calif.). MAbs reactive with CXCR4 (12G5), CCR5 (3A9), CD45 (HI30), CD55 (1A10), CD80 (L307.4), CD86 (IT2.2), and MHC-I (G46-2.6) were all purchased from Pharmingen (San Diego, Calif.). All antibodies were phycoerythrin coupled. Following antibody staining, cells were washed three times with 250 µl of staining buffer and fixed with 2% paraformaldehyde overnight at 4°C prior to data acquisition on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson Immunocytometry Systems). Samples were gated on viable cells by forward and 90° light scatter, and at least 15,000 live-cell events were acquired for each sample. Acquired data were analyzed by using FlowJo software (Tree Star, Inc., San Carlos, Calif.).

Western blot analysis. Cells, virions, and microvesicles were solubilized in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.05 M Tris hydrochloride buffer [pH 7.5], 0.15 M NaCl, 1 mM EDTA, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were cleared by microcentrifugation at 12,000 × *g* for 5 min at 4°C. HIV-1 virions and microvesicles (50 µg of total protein equivalents per lane) for electrophoresis were run separately on discontinuous SDS-polyacrylamide (4 to 20% gradient) gels under nonreducing or reducing conditions. Proteins were transferred onto Immobilon-P membranes by a semidry blotting technique (Millipore, Bedford, Mass.), and specific proteins were detected by immunoblot analysis with a MAb against CD45 (HI30; Pharmingen), a rabbit polyclonal Ab to CD55 (H-319; Santa Cruz Biotechnology, Santa Cruz, Calif.), a goat polyclonal IgG against CD80 (N-20; Santa Cruz Biotechnology), a mouse MAb to CD86 (IT2.2; Pharmingen), a mouse MAb to MHC-I (a generous gift from Hidde Ploegh, Massachusetts Institute of Technology, Cambridge), or a mouse MAb to MHC-II (L243; American Type Culture Collection). Primary antibodies were detected with horseradish peroxidase-conjugated, species-specific goat secondary antibodies (Bio-Rad, Hercules, Calif.) and enhanced-chemiluminescence reagents (Amersham, Arlington Heights, Ill.).

VPA. A whole-virion immunoprecipitation assay (VPA) was performed essentially as previously described (2, 40), except that it was performed with a 96-deep-well (2.2 ml) plate (Marsh Biomedical Products, Inc., Rochester, N.Y.) or microcentrifuge tubes. Comparable input amounts of infectious or Aldrichol-2-inactivated virus preparations (p24^{CA} at 10,000 pg/ml or reverse transcriptase equivalents at 2,500 pg/ml) were incubated overnight at 4°C on a rocker with each MAb at 10 µg/ml in PBS plus 3% bovine serum albumin (BSA) in a total volume of 500 µl in deep-well plates sealed with aluminum plate sealers (Beckman, Fullerton, Calif.). Pansorbin cells (formalin-fixed *Staphylococcus aureus* strain Cowan; 25 µl; Calbiochem, La Jolla, Calif.) were incubated with PBS-3% BSA or with rabbit anti-mouse IgG (Sigma) under saturating conditions and washed three times in PBS plus 3% BSA. Pansorbin-Ab complexes were added directly to virus complexed with the mouse MAbs, and after incubation at 20°C for 30 min with rocking, virion Ab-Pansorbin complexes were precipitated by centrifugation (2,000 × *g*, 30 min). The residual virus content of the supernatant after immunoprecipitation was determined by p24 capture immunoassay (AIDS Vaccine Program, NCI at Frederick) or reverse transcriptase assay (Cavidi). The MAbs used in the VPA were the same MAbs used in the flow cytometry exper-

iments. Clearance by a particular Ab in this assay is indicative of the presence of immunoreactive antigens on the virion surface (2). It is likely that a threshold density of a host cell-derived protein in the virus envelope is required to precipitate the virus and that the amount of virus precipitated depends in part on the density of a given protein in the envelope of the virus. However, because the VPA readout involves quantitation of a viral protein, this assay measures how many virions have been precipitated by the Ab-Pansorbin complex and not the number of host cell-derived proteins on a virion. Adding a rabbit anti-mouse secondary Ab to the Pansorbin cells allowed us to detect CD80 on virions that appeared to be CD80 negative when precipitated with the anti-CD80 MAb alone (D.G., unpublished observation). Error bars represent 1 standard deviation of the mean of triplicate measurements. *P* values were calculated by using a one-tailed, equal-variance Student *t* test of experimental measurements versus isotype control measurements. Statistical analysis was performed by using Microsoft Excel. Proteins for which immunoprecipitation with a specific MAb yielded a value statistically significantly greater than the value for the isotype control were considered to be incorporated into the virions at significant levels.

HLA-DR genotyping. DRB1 genotyping was performed by using a combination of PCR sequence-specific priming (31) and single strand-strand conformation polymorphism (8) analyses.

RESULTS

Differential incorporation of CD80, CD86, MHC-I, and MHC-II into HIV-1 virions. HIV preferentially incorporates or excludes different host cell proteins when budding from an infected cell. We have hypothesized that the presence of MHC molecules or the costimulatory protein CD80 or CD86 in the HIV-1 virion envelope could contribute to HIV pathogenesis (14). HIV incorporates MHC-I and MHC-II upon budding from infected T cells or macrophages *in vitro* (2, 6, 9) and *in vivo* (26, 27, 41), but it had previously not been determined whether the costimulatory proteins CD80 and CD86 are also incorporated into the HIV-1 virion envelope. By using a sensitive, Ab-based VPA, we performed an initial survey of seven primary HIV isolates derived from PBMC, two MΦ-derived isolates, and three laboratory isolates to determine whether CD80, CD86, MHC-I, and MHC-II were incorporated into the virions. All of the virions incorporated MHC-II, except the virions derived from the MHC-II-negative CEMX174/T2 cell line (Fig. 1). None of the virions incorporated significant levels of CD80, and 9 of the 12 viruses incorporated CD86 (Fig. 1). There was variable incorporation of MHC-I into the virions, depending on the virus and the cells from which the virus was produced (Fig. 1). These data suggested that, depending on the virus and the cell from which it was derived, there could be differential incorporation of CD80, CD86, MHC-I, and MHC-II into the virion envelope and that CD86 was more readily incorporated into virions than was CD80.

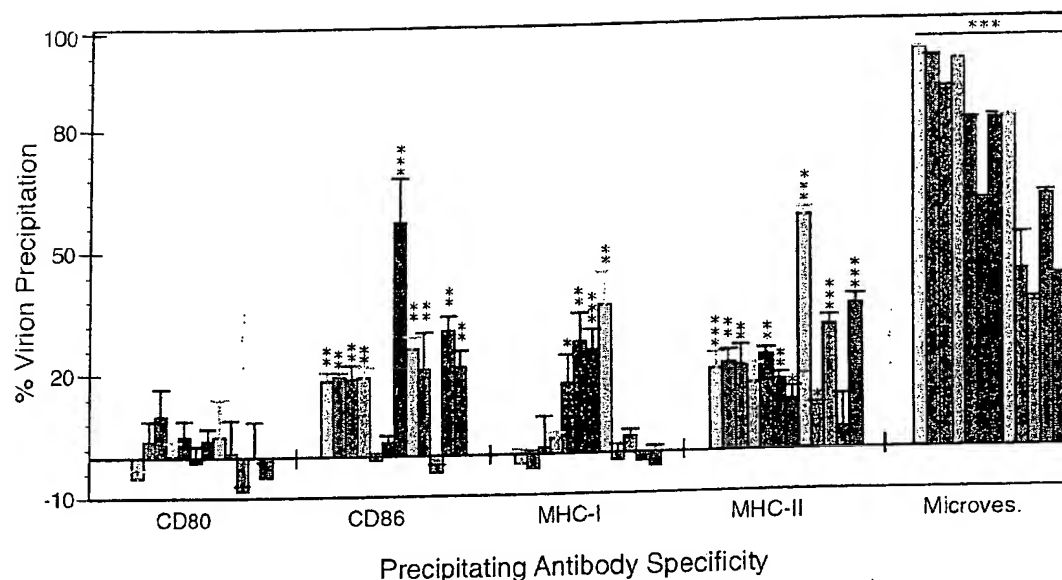
We attempted to determine the basis for the differential presence of different host cell proteins in different virus preparations produced from different cell types. To determine whether the presence or absence of CD80, CD86, MHC-I, and MHC-II on virions is directly related to the levels of these molecules on the surface of the cells from which the virus was produced, we examined the levels of these molecules on the surface of uninfected cells and that of the HIV-1-infected cells from which the virus we studied was produced. In addition to measuring the levels of CD80, CD86, MHC-I, and MHC-II on the uninfected and infected cells, we also examined the levels of CD45 and CD55. CD45 is one of the most highly expressed proteins on the surfaces of lymphocytes and monocytes and is reportedly excluded from virions produced from the Jurkat T-cell line (29). CD55 is a glycosylphosphatidylinositol-linked

protein that is localized to cholesterol-rich regions in the plasma membrane, termed rafts, and its incorporation into virions has been used as evidence for virion budding through rafts (28). We therefore characterized the cell surface expression of CD4, CXCR4, CCR5, CD45, CD55, CD80, CD86, MHC-I, and MHC-II on uninfected and HIV-1-infected MΦ (see Table 1), PBMC (see Table 2), and cell lines (see Table 3) and characterized the incorporation of CD45, CD55, CD80, CD86, MHC-I, and MHC-II into HIV-1 virions derived from MΦ (see Fig. 2), PBMC (see Fig. 3), and cell lines (see Fig. 4 and 5), respectively.

Profile of immunoregulatory molecules incorporated into MΦ-derived HIV-1 virions. Monocyte-derived MΦ expressed low to moderate levels of CD4 and both coreceptors CXCR4 and CCR5 (Table 1). MΦ infected with Ada-M, Ba-L 98-4, and Ba-L 98-7 showed increased expression of CD45, CD55, CD86, MHC-I, and MHC-II (Table 1). The uninfected and infected MΦ expressed low levels of CD86 and low to undetectable levels of CD80 (Table 1). Characterization of the proteins incorporated into the MΦ-derived virions revealed that CD80 was detectable on the Ba-L 98-7 and Ada-M 98-3 virions and CD86 was detectable on the Ba-L 98-4, Ba-L 98-7, and Ada-M 98-3 virions. Interestingly, the MΦ-derived virions did not incorporate detectable amounts of CD45 or CD55 (Fig. 2), despite moderate levels of CD45 and CD55 expression on the MΦ (Table 1). Lastly, all three MΦ tropic viruses incorporated significant levels of MHC-I and MHC-II (Fig. 2). These data support the premises that MHC-II is preferentially incorporated into MΦ-derived virions and that CD55 and CD45 are preferentially excluded.

Profile of immunoregulatory molecules incorporated into PBMC-derived HIV-1 virions. We next characterized the cell surface expression and incorporation of cell surface proteins with immunoregulatory function into representative X4, R5, and dual-tropic HIV-1 virions produced from primary PBMC. The levels of CD4, CXCR4, CCR5, CD45, CD55, CD80, CD86, MHC-I, and MHC-II on activated PBMC revealed that the majority of the cells were CD4 and CXCR4 positive and CCR5 negative (Table 2). The majority of the cells expressed low levels of CD80 and moderate levels of CD55, CD86, and MHC-I. As observed with the MΦ, CD45 was the most highly expressed molecule and cell surface MHC-II expression was increased by HIV-1 infection (Table 2).

Characterization of the immunoregulatory proteins incorporated into the representative R5-tropic (SF162), dual-tropic (89.6), and X4-tropic (NL4-3) virions produced from PBMC revealed that CD80 was present on the SF162 virions but not on the 89.6 or NL4-3 virions (Fig. 3). All three PBMC-derived viruses incorporated significant levels of CD55, CD86, and MHC-II (Fig. 3). The SF162 virions and the NL4-3 virions incorporated significant levels of MHC-I, but the 89.6 virions did not (Fig. 3). As observed for the MΦ-produced virions, CD45 was not incorporated into the PBMC-derived virions (Fig. 3), despite being the most highly expressed molecule on the surface of the HIV-infected PBMC (Table 2). These data further supported the hypotheses that HIV infection upregulates MHC-II cell surface expression and that MHC-II is preferentially incorporated into budding virions whereas CD45 is preferentially excluded.



108-436/PBMC	115/PBMC	91US054/PBMC	MN/H9
2-285/PBMC	92US657/PBMC	Ada-M/MΦ	NL4-3/T2
P419/PBMC	92US727/PBMC	Ba-L/MΦ	NL4-3/TBLCL-CD4

FIG. 1. Survey analysis of differential incorporation of CD80, CD86, MHC-I, and MHC-II into virions of a panel of HIV-1 isolates propagated in PBMC, MΦ, or cell lines. Immunoprecipitation of primary HIV-1 isolates, MΦ-tropic isolates, and cell line-adapted virions was performed by using a MAb-based VPA as described in Materials and Methods. MAbs to immunoregulatory proteins CD80, CD86, MHC-I, and MHC-II were used to characterize primary isolates (108-436, 2-285, P419, 115, 92US657, 92US727, and 91US054 [blue shades]), MΦ isolates (Ada-M 98-4 and Ba-L 98-6 [red shades]) and cell line-adapted isolates (MN/H9, NL4-3/T2, and NL4-3/TBLCL-CD4 [green shades]). A polyclonal antiserum raised against microvesicles derived from the TBLCL-CD4 cell line served as a positive control for maximal virion precipitation, and an isotype-matched mouse anti-CD4 MAb served as a negative control. The data shown are representative of two independent experiments performed in triplicate with <10% variability in the magnitude of virion clearance. Error bars represent 1 standard deviation of the mean of triplicate measurements. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's t -test significance of differences between experimental measurements and isotype control measurements).

Comparison of the profiles of immunoregulatory molecules incorporated into cell line-derived HIV-1 virions and microvesicles. We next sought to characterize and compare the immunoregulatory proteins incorporated into HIV-1 virions and microvesicles. Microvesicles are nonviral membrane vesicle particles of unknown biological and immunological signifi-

cance that bud from the surface of cells (5, 18). Identification and quantitation of cellular proteins associated with HIV-1 virions have been complicated by the presence of these microvesicles that inevitably copurify with HIV virions (5, 18). We have previously shown that microvesicles contain high levels of β 2-microglobulin, MHC-I, and MHC-II (5), but it had

TABLE 1. Cell surface expression of cellular proteins on uninfected and HIV-1-infected MΦ*

Cell type	Virus strain	Isotype control	CD4	CXCR4	CCR5	CD45	CD55	CD80	CD86	MHC-I	MHC-II
MΦ		31	78	32	49	333	101	9	43	103	140
MΦ	Ada-M98-3	27	59	31	53	479	109	12	75	168	300
MΦ	Ba-L98-4	23	34	30	45	470	121	14	86	153	311
MΦ	Ba-L98-7	19	9	27	42	372	108	14	125	166	342

* MΦ were isolated and either mock infected or infected with Ada-M98-3, Ba-L98-4, or Ba-L98-7 as described in Materials and Methods. After 18 days, cell surface expression of CD4, CXCR4, CCR5, CD45, CD55, CD80, CD86, MHC-I, and MHC-II was determined by flow cytometric analysis. Mean fluorescence intensity values (in arbitrary fluorescence units) for cell surface expression of the various proteins are presented. The data shown are representative of two separate experiments.

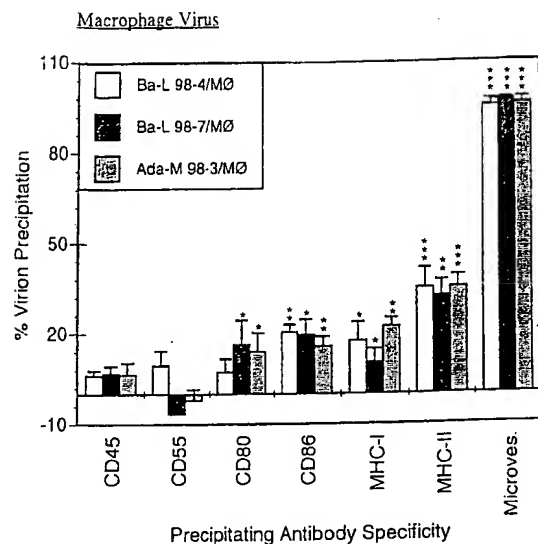


FIG. 2. Profile of immunoregulatory molecules incorporated into HIV-1 virions produced from infected MΦ. MΦ were isolated and cultured as described in Materials and Methods. MΦ were mock infected or infected with Ada-M 98-3, Ba-L 98-4, or Ba-L 98-7. MΦ-derived virions were characterized for the presence of CD4, CD45, CD55, CD80, CD86, MHC-I, and MHC-II in the virion envelope. The data shown are representative of two separate experiments, each performed in triplicate. Error bars represent 1 standard deviation of the mean of triplicate measurements. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t*-test significance of differences between experimental measurements and isotype control measurements). Microves., microvesicles.

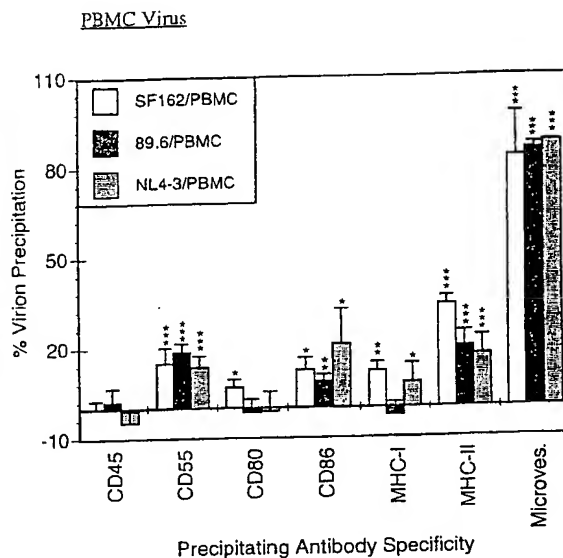


FIG. 3. Profile of immunoregulatory molecules incorporated into HIV-1 virions produced from infected PBMC. PBMC were isolated and cultured as described in Materials and Methods. PHA- and IL-2-activated PBMC were mock infected or infected with CCR5-tropic SF162, dual-tropic 89.6, or CXCR4-tropic NL4-3. PBMC-derived virions were characterized for the presence of CD4, CD45, CD55, CD80, CD86, MHC-I, and MHC-II in the virion envelope. The data shown are representative of two separate experiments, each performed in triplicate. Error bars represent 1 standard deviation of the mean of triplicate measurements. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t*-test significance of differences between experimental measurements and isotype control measurements). Microves., microvesicles.

not been previously determined whether microvesicles contain CD45, CD55, CD80, or CD86.

To determine if these immunoregulatory molecules are incorporated into microvesicles or HIV-1 virions, we first examined their cell surface expression on four different cell lines used to produce HIV-1_{NL4-3}. Flow cytometric analysis of uninfected cultures of the T1, T2, TBLCL-CD4, and H9 cell lines and parallel infected cultures revealed that the four cell lines expressed CXCR4, but not CCR5, and expressed moderate to high levels of CD4, CD45, CD55, MHC-I, and MHC-II (Table 3). The H9 T-cell line did not express CD80 or CD86, and as

expected (44), the T2 cell line did not express MHC-II and expressed very low levels of MHC-I (Table 3). CD80 and CD86 were expressed at higher levels on the T1, T2, and TBLCL-CD4 cell lines than on MΦ or freshly isolated PBMC (Table 3). Cell surface MHC-II expression was increased by HIV infection on the H9 and TBLCL-CD4 cell lines but not on the T1 cell line.

We next examined matched microvesicle and virion preparations by Western blot analysis to determine whether CD45, CD55, CD80, CD86, MHC-I, or MHC-II was present in the

TABLE 2. Cell surface expression of cellular proteins on uninfected and HIV-1-infected PBMC^a

Cell type	Virus strain	Isotype control	CD4	CXCR4	CCR5	CD45	CD55	CD80	CD86	MHC-I	MHC-II
PBMC	None	4	21	45	4	3,831	432	11	352	117	726
PBMC	Ada-M (R5) ^c	3	48	64	5	5,221	735	18	212	303	1,271
PBMC	Ba-L (R5) ^c	6	3	65	8	4,818	657	9	312	234	1,160
PBMC	JRFL (R5) ^c	3	2	48	4	4,984	620	22	257	219	1,021
PBMC	SF162 (R5) ^b	4	3	24	4	4,052	347	8	358	111	812
PBMC	89.6 (X4R5) ^b	7	3	36	5	4,909	667	15	388	253	1,254
PBMC	NL4-3 (X4) ^b	2	3	203	6	4,054	625	23	270	162	956

^a PBMC were isolated and prepared as described in Materials and Methods. Activated PBMC were infected with either CXCR4-tropic HIV-1 isolate NL4-3; CCR5-tropic HIV-1 isolate Ada-M, Ba-L, JRFL, or SF162; or dual-tropic 89.6. After 7 days, the PBMC were analyzed by flow cytometry for CD4, CXCR4, CCR5, CD45, CD55, CD80, CD86, MHC-I, and MHC-II expression. Mean fluorescence intensity values (in arbitrary fluorescence units) for cell surface expression of various proteins are presented.

^b This virus strain was characterized for the cellular proteins incorporated into the virions by the VPA.

^c Infection of PBMC with this virus strain did not produce enough virus for characterization by the VPA.

TABLE 3. Cell surface expression of cellular proteins on uninfected and acutely HIV-1_{NL4-3}-infected cell lines^a

Cell type	Virus strain	Isotype control	CD4	CXCR4	CCR5	CD45	CD55	CD80	CD86	MHC-I	MHC-II
H9		3	85	281	8	2,665	206	5	9	228	1,418
H9	NL4-3	4	142	170	5	2,599	209	7	9	265	3,938
T1		3	445	233	5	1,452	453	707	478	351	2,124
T1	NL4-3	3	8	112	11	2,173	733	607	597	187	2,026
T2		8	662	206	9	1,628	358	907	778	95	34
T2	NL4-3	9	6	196	8	1,323	708	933	1,513	30	12
TBLCL		8	75	62	7	1,797	316	528	402	302	4,162
TBLCL	NL4-3	9	7	32	8	1,418	194	310	214	318	5,178

^a Cell lines were prepared as described in Materials and Methods. Cell lines were acutely infected with HIV-1_{NL4-3}, and cell surface expression of CD4, CXCR4, CCR5, CD45, CD55, CD80, CD86, MHC-I, and MHC-II was examined by flow cytometry on day 7 postinfection. Mean fluorescence intensity values (in arbitrary fluorescence units) for cell surface expression of various proteins are presented. The data shown are representative of two separate experiments.

preparations. Microvesicles and HIV-1 virions derived from the T1, T2, TBLCL-CD4, and H9 cell lines were purified by sucrose banding density centrifugation and quantitated for total protein and p24 capsid levels. TBLCL-CD4 cell lysate served as a positive control because the TBLCL-CD4 cell line expressed moderate to high levels of CD45, CD55, CD80, CD86, MHC-I, and MHC-II (Table 3). Immunoblot analysis revealed that both the virion and microvesicle preparations contained large amounts of CD45, CD55, CD86, MHC-I, and MHC-II but not CD80 (Fig. 4). CD80 was readily detected in as little as 5 µg of total TBLCL-CD4 cell lysate but was barely detectable in 50 µg of the T1, T2, or TBLCL-CD4 virion or microvesicle preparations, suggesting that CD80 was excluded from both virions and microvesicles (Fig. 4). In contrast to CD80, CD86 was weakly detected in the TBLCL-CD4 cell lysate but easily detected in virion and microvesicle preparations, suggesting that CD86 was preferentially incorporated into virion and microvesicle preparations (Fig. 4). Neither CD80 nor CD86 was detected in the H9 virion or microvesicle preparations due to the fact that the H9 cell line did not express CD80 or CD86 (Table 3). Per microgram of total protein, there was more MHC-I and MHC-II in the microvesicle and virion preparations than in the cell lysate, suggesting that both microvesicle and virion preparations were enriched for MHC-I and MHC-II (Fig. 4). Importantly, CD45 was present at high levels in both the microvesicle and virion preparations. These findings demonstrate that microvesicle and virion preparations contained high levels of CD45, CD55, CD86, MHC-I, and MHC-II but that CD80 was excluded or present at very low levels.

As noted previously, even sucrose-banded HIV-1 virion preparations still contain copurifying microvesicles (5, 18). Because immunoblot analysis of the virion preparations (Fig. 4) cannot distinguish between virion-associated and microvesicle-associated host cell-derived molecules, we determined whether CD45, CD55, CD80, CD86, MHC-I, or MHC-II was incorporated into HIV-1_{NL4-3} virions derived from the T1, T2, TBLCL-CD4, and H9 cell lines by using the VPA. In this assay format, antibodies to host cell proteins incorporated into virions immunoprecipitate the virions while antibodies to host cell proteins present in virion preparations, but not physically incorporated into viral particles, for example, in copurifying microvesicles in the preparations, do not immunoprecipitate virions. Based on this immunoprecipitation assay, CD55 was significantly present on virions from all four sources (Fig. 5).

MHC-I and MHC-II were significantly detected on virions derived from T1, TBLCL-CD4, and H9 cells but not on those from T2 cells (Fig. 5). CD86, but not CD80, was detected on virions derived from CD80 and CD86-expressing cells (Fig. 5), despite equivalent levels of CD80 and CD86 on the surfaces of the T1, T2, and TBLCL-CD4 cells (Table 3). Additionally, the anti-CD45 MAb did not precipitate virions derived from any of the cell lines (Fig. 5), although CD45 is the most highly expressed protein on the surfaces of all four cell lines (Table 3). These data extend the previous finding that there can be preferential incorporation of MHC-II and CD86 and preferential exclusion of CD80 and CD45. Importantly, these data reveal that the CD45 detected on the virions by Western blot analysis was present on the copurifying microvesicles and not incorporated into the virions.

Host cell-derived HIV-1 virion-associated proteins affect virion-triggered cell death. As described in this report and elsewhere, HIV incorporates MHC molecules when it buds from infected cells. We have postulated that virion-associated, host cell-derived proteins might play a role in HIV pathogenesis (2), but previously it has been difficult to distinguish between cell death due to the direct effects of viral replication and lysis from indirect effects due to noninfectious virions. Specifically, we have proposed that MHC molecules incorporated into the HIV virion can interact with the TCR and other receptors on the surface of a T lymphocyte to induce anergy or apoptosis (2, 39). We have recently developed a procedure by which to inactivate HIV infectivity without affecting the conformational integrity of the virion surface proteins (1, 40). These conformationally and functionally intact but noninfectious virions interact authentically with target cells and provide a powerful tool with which to evaluate the role host cell-derived proteins present on the HIV-1 virion play in pathogenesis, independently of productive infection.

To better understand the effect of virion-associated host cell-derived proteins in HIV pathogenesis, we examined the effects of microvesicles and conformationally authentic, noninfectious HIV-1_{NL4-3-ΔT2} virions produced from T1, T2, TBLCL-CD4, and H9 cells on freshly isolated PBMC from a healthy, HIV-seronegative donor. Microvesicles derived from the four cell lines did not induce cell death in the cultures (Fig. 6). CD86-positive, MHC-positive, noninfectious virions derived from the CEMX174/T1 cell line triggered cell death, whereas CD86-positive, MHC-negative, noninfectious virions derived from the matched, MHC-II-negative CEMX174/T2

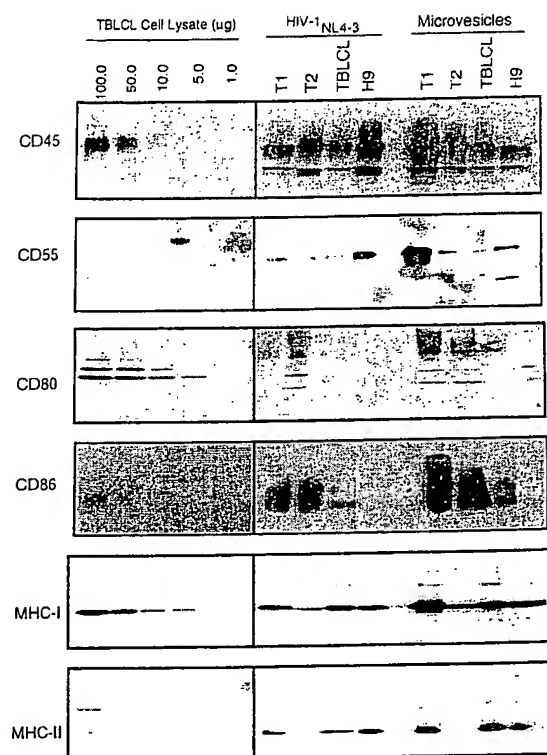


FIG. 4. Virion and microvesicle preparations contain high levels of CD45, CD55, CD86, MHC-I, and MHC-II but not CD80. Virions and microvesicles derived from the T1, T2, TBCLCL-CD4, and H9 cell lines were purified by sucrose density gradient ultracentrifugation. TBCLCL-CD4 cell lysates served as a positive control and a way to determine the sensitivities of the different antibodies in the Western blot assays. Virion and microvesicle preparations (50 μ g of total protein per lane) and the TBCLCL-CD4 lysates were analyzed on an SDS-5 to 20% nondenaturing polyacrylamide gel under reducing or nonreducing conditions. Immunoblots were probed with a MAb to CD45 (H130), a polyclonal serum to CD55 (H-319), a goat polyclonal serum to CD80 (N-20), a MAb to CD86 (IT2.2), a MAb to MHC-I, and a MAb to MHC-II (L243). The results shown are representative of at least three independent Western blot assays for each protein.

cell line did not (Fig. 6). Because these two cell lines differ only in MHC expression, these data strongly suggest that virion-associated MHC molecules can impact HIV pathogenesis. The CD86-positive, MHC-positive, noninfectious virions derived from the TBCLCL-CD4 cell line also triggered cell death (Fig. 6). However, the MHC-positive, CD86-negative, noninfectious virions derived from the H9 cell line did not trigger cell death (Fig. 6). The differential killing effect of noninfectious HIV-1_{NL4-3} virions derived from different cell lines suggests that immunoregulatory proteins incorporated into the HIV virion, such as CD86, MHC-I, and MHC-II, may contribute importantly to indirect mechanisms of HIV pathogenesis.

DISCUSSION

A hallmark of HIV infection is the functional impairment of CD4⁺ T lymphocytes that precedes an eventual decline in

circulating CD4⁺ T cells. The mechanism(s) behind this HIV-induced unresponsiveness or "anergy" and eventual apoptosis of CD4⁺ T cells remains unclear. Here we propose that host cell-derived immunoregulatory proteins present in the envelope of noninfectious virions could impact HIV pathogenesis. Specifically, binding of gp120 to CD4, virion-associated MHC molecules to TCRs, and virion-associated CD86 to CD28 on T lymphocytes could lead to T-cell activation, differentiation, anergy, or apoptosis. T cells normally require two signals to become fully activated. Signal one is Ag specific and is initiated by TCR binding to Ag-MHC complexes on the APC. The second, or costimulatory, signal is generated by CD28 on the T cell binding to CD80 or CD86 on the APC (reviewed in reference 19). We therefore determined whether HIV-1 virions or microvesicles incorporate CD80 or CD86. When a sensitive immunoprecipitation procedure was used, CD80 was detected on only 3 of 21 viruses derived from CD80-expressing cells whereas CD86 was detected on 17 of 21 viruses derived from CD86-expressing cells (Fig. 1, 2, 3, and 5). Additionally, virions and microvesicles derived from the T1, T2, and TBCLCL-CD4 cells preferentially incorporated CD86 compared to CD80 (Fig. 4 and 5), despite approximately equivalent levels of CD80 and CD86 expression on the three cell lines (Table 3). These results suggest that CD86 is generally incorporated into budding virions and microvesicles, whereas CD80 is generally excluded. The molecular mechanisms behind the preferential incorporation of CD86 and exclusion of CD80 remain to be elucidated, but this phenomenon could be mediated by the cytoplasmic domains, which bear no similarity to one another (3).

The immunological significance of microvesicles enriched for MHC-I, MHC-II, and CD86 but not CD80 is also unclear. However, in some experiments, microvesicles have suppressed virus-specific T-cell responses (M. T. Esser, unpublished observation). CD80 and CD86 do not simply play redundant roles in the immune system (19). Antibodies that bind CD86 block the development of Th2 T cells and can exacerbate inflammation, whereas Abs that bind CD80 can reduce the severity of inflammation in certain models of autoimmunity (24, 37). These and other studies raise the possibility that interactions with CD86 present on virions and microvesicles may help differentiate naive T cells into Th2-like effectors (11, 46). Interestingly, there is an increase in the percentage of CD86-expressing CD4⁺ T lymphocytes in HIV-infected individuals (A. Valentin, personal communication). Microvesicles may be a mechanism the immune system uses to down-regulate ongoing inflammatory responses. HIV and other viruses may have exploited this microvesicle secretion pathway as a way to enhance virion assembly and as a mechanism to suppress a T-cell-mediated immune response.

We also undertook these studies in the hope of identifying a protein present on microvesicles that was not present on HIV-1 virions. As mentioned previously, microvesicles can be roughly the same size as HIV virions and band at the same density (1.13 to 1.16 g/ml) as HIV-1 virions in a sucrose gradient and are an inevitable contaminant of all HIV-1 preparations (5, 18). Toward this end, we identified CD45 as a molecule that was present at high concentrations on microvesicles but was not detected on virions (Fig. 1, 2, 3, and 5), despite being the most highly expressed protein on all of the cells

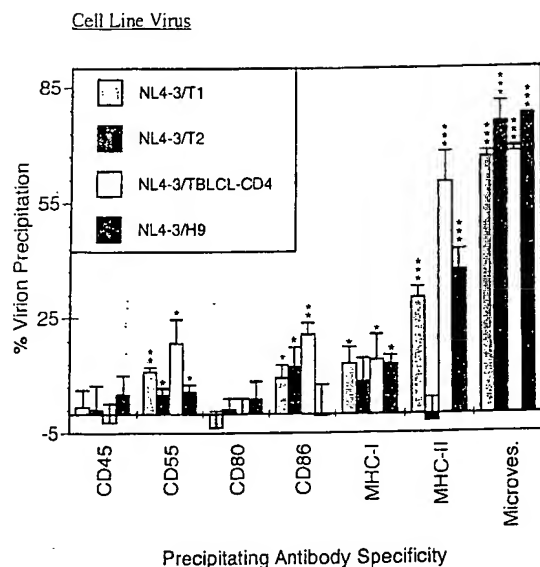


FIG. 5. Profile of immunoregulatory molecules incorporated into HIV-1 virions produced from chronically infected continuous cell lines. Chronically infected cell lines were maintained in culture as described in Materials and Methods. Cell-free HIV-1_{NL4-3} derived from the T1, T2, TBLC-CD4, and H9 cell lines was purified by sucrose density gradient ultracentrifugation. The purified virion preparations were characterized for the presence of CD4, CD45, CD55, CD80, CD86, MHC-I, and MHC-II in the virion envelope. The data shown are representative of three separate experiments, each performed in triplicate. Error bars represent 1 standard deviation of the mean of triplicate measurements. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t*-test significance of differences between experimental measurements and isotype control measurements). Microves., microvesicles.

examined (Tables 1, 2, and 3). These results extend the findings of Nguyen and Hildreth that CD45 was not incorporated into HIV-1_{RF} derived from the Jurkat T-cell line (29). Importantly, the presence of CD45 on microvesicles but not on virions may provide a way in which to purify HIV-1 virions of contaminating microvesicles. Microvesicle-free HIV-1 preparations would have practical applications for biochemical analyses. The ability to remove microvesicles from purified virus preparations may also be advantageous for the production of inactivated HIV-1 vaccines.

The mechanism(s) that determines which proteins are incorporated into the budding HIV-1 virion is not well understood. The incorporation of immunoregulatory proteins into virions was not random, since some highly expressed proteins, like CD45, were excluded from virions while others, like MHC-II, appeared to be specifically incorporated (Fig. 1, 2, 3, and 5). Nguyen and Hildreth have proposed that HIV-1 buds selectively from glycolipid-enriched membrane domains called lipid rafts (29). Supporting this hypothesis, we found the lipid raft marker CD55 on T-cell-derived virions, suggesting that the T-cell-tropic virions budded from these rafts whereas the CD55-negative, MΦ-tropic virions may have budded via a different mechanism. In this regard, it is worth noting that in HIV-1-infected MΦ virions accumulate in intracellular vacu-

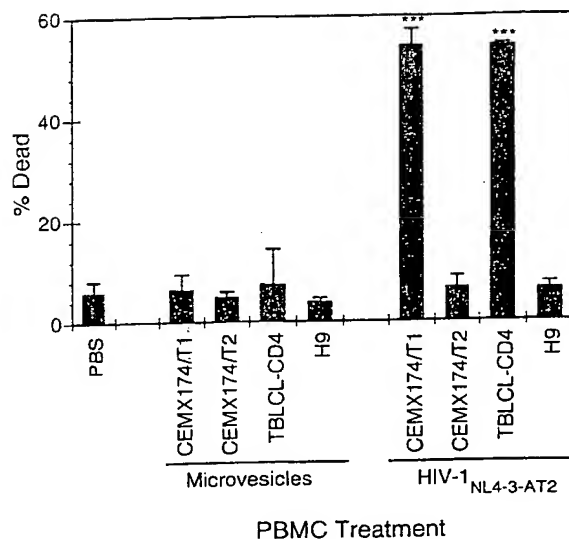


FIG. 6. Virion-associated cellular molecules play a role in virion-triggered cell death. The effect of virion-associated, host cell-derived molecules in HIV pathogenesis was examined by using conformationally authentic, noninfectious HIV-1 virions. Noninfectious, Aldrichol-2-inactivated virions (p24^{CA} equivalents at 50 ng/ml) or microvesicles (total protein at 10 μg/ml) were used to pulse resting PBMC from a healthy, HIV-seronegative donor. After 10 days, the PBMC were enumerated for total cell numbers and percent viability by trypan blue analysis. The data shown are representative of three separate experiments. Error bars represent 1 standard deviation of the mean of triplicate measurements. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t*-test significance of differences between experimental measurements and PBS [control] measurements).

oles and are rarely seen budding from the plasma membrane (32) whereas T-cell-derived viruses bud predominantly from the plasma membrane (22, 23). CD55 may be a useful marker with which to dissect the different pathways that MΦ-tropic and T-cell-tropic virions use to egress from an infected cell. It is also possible that HIV-1-encoded proteins directly bind to host cell proteins to facilitate their incorporation into the mature virion. We have previously reported that a 43-amino-acid region in the cytoplasmic tail of gp41 is required for the efficient incorporation of MHC-II, but not MHC-I, into HIV-1 virions derived from T-cell lines and PBMC (36). These results suggest that HIV-1 may have evolved to specifically incorporate MHC-II into the virion as a mechanism of immune evasion.

Regardless of the specific mechanism that HIV uses to incorporate host cell-derived proteins, it is clear from the experiments with HIV-1_{NL4-3-AT2} virions that host cell-derived proteins can dramatically affect viral pathogenicity (Fig. 6). The HLA-DR genotype of the T1, T2, TBLC-CD4, and H9 cell lines; the phenotype of the virion envelope; and whether the HIV-1_{NL4-3-AT2} virions triggered cell death are summarized in Table 4. MHC-containing, T1-derived virions triggered cell death, whereas MHC-negative, T2-derived virions did not (Fig. 6 and Table 4), strongly suggesting that virion-associated MHC molecules contribute to HIV pathogenesis. Importantly, the

TABLE 4. Virion-associated cellular proteins contribute to HIV-1 pathogenesis in vitro^a

Cell type	HLA-DRB1, DRB2 genotype	Virus strain	Virion-associated cellular proteins						Cell death
			CD45	CD55	CD80	CD86	MHC-I	MHC-II	
CEMX174/T1	0701, 0701	NL4-3	—	+	—	+	+	+	+
CEMX174/T2	None	NL4-3	—	+	—	+	+	+	+
TBLCL-CD4	1501, 1104	NL4-3	—	+	—	—	+	+	—
H9	0400, 0400	NL4-3	—	+	—	—	+	+	—

^a The HLA-DR beta genotype of the T1, T2, TBLCL-CD4, and H9 cell lines was determined by a combination of PCR sequence-specific priming (31) and single strand-strand conformation polymorphism (8) analyses. The HLA-DR genotype of the PBMC donor was 1101, 0701. The presence (+) or absence (—) of the immunoregulatory proteins on the HIV-1_{NL4-3-AT2} virions produced from the four cell lines is shown. Virion-triggered cell death data from the experiment shown in Fig. 6 are summarized as either positive (+) or negative (—), with positivity defined as 30% dead cells as determined by trypan blue uptake at day 10 of culture following exposure to AT2-inactivated, noninfectious HIV-1_{NL4-3-AT2} virions.

T1- and T2-derived virions differed only in MHC expression (Fig. 5 and Table 4) due to the fact that the T2 cell line has a deletion in chromosome 6 (44). Interestingly, AT2-inactivated NL4-3/H9 virions did not trigger cell death despite containing MHC-II (Fig. 6). This may have been due to the fact that H9-derived virions did not contain CD86, whereas the T1- and TBLCL-CD4-derived virions did (Fig. 5 and Table 4), or it may have been due to the fact that the H9-derived virions contained HLA-DRβ 0400, whereas the T1-derived virions contained HLA-DRβ 0701 and the TBLCL-CD4-derived virions contained HLA-DRβ 1501, 1104 (Table 4). Future studies will determine whether the HLA-DR phenotype of the virus or the responder PBMC affects HIV-1-triggered apoptosis.

Virion-associated MHC molecules could play several roles in HIV pathogenesis. The natural ligands for MHC-II are the TCR and CD4, and virion-associated MHC-II could enhance the avidity of the virion to increase infectivity as reported by Cantin et al. (6). Alternatively, virion-associated MHC-I and MHC-II could bind to TCRs on CD8⁺ and CD4⁺ T lymphocytes, respectively, to trigger apoptosis. Because the AT2-inactivated virions were not infectious, our data favor the second interpretation. Noninfectious-virion-triggered cell killing is especially relevant in the light of recent data from Lawn and Butera demonstrating that virions isolated from patient plasma during primary viremia did not contain MHC-II molecules whereas virions isolated late in infection or from patients with opportunistic infections contained high levels of MHC-II (26). Our current evidence supports the hypothesis that HIV has evolved specific strategies by which to acquire MHC-II as a way to thwart the host immune response.

In summary, this study demonstrated that the incorporation of host cell proteins into virions and microvesicles was not random. HIV-1 infection of MΦ, PBMC, and cell lines increased cell surface expression of MHC-II, and all of the viruses examined incorporated MHC-II. CD86, but not CD80, was preferentially incorporated into both microvesicles and virions. CD45 was identified as a molecule that was highly expressed on microvesicles but excluded from virions. Studies with noninfectious HIV-1_{NL4-3-AT2} virions revealed that host cell-derived proteins can dramatically affect the pathogenicity of HIV-1 virions. Dissection of the mechanisms by which HIV acquires host cell immunoregulatory proteins and the role virion-associated host cell proteins play in triggering cell death will advance our understanding of HIV pathogenesis.

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EXHIBIT D

Transient transfection of ecotropic retrovirus receptor permits stable gene transfer into non-rodent cells with murine retroviral vectors

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Retroviral vectors are useful for efficient, stable and single copy transfer of foreign genes into animal cells. However, the low risk murine ecotropic vectors can only be used with rodent cells due to their restricted host range. When using cells from other species, in particular human, amphotropic retroviral vectors and the corresponding packaging cell lines are required. Given the wide host range of these vectors, they are classified as higher biological risk and high biological containment (L2 or even L3) is mandatory in many countries. To reduce the risk to the experimenter while working with non-rodent cells, we considered a strategy based on transient expression of the recently cloned murine ecotropic retrovirus receptor (1) to allow the use of safer murine retroviral vectors.

Murine retroviruses enter target cells by interaction of the viral envelope glycoprotein with a specific cell-surface receptor, a basic amino acid transporter (2,3). The retroviral host range is determined by the species- and/or tissue-specific expression of the appropriate receptor. To extend the host range of the rodent-specific Moloney murine leukemia virus we transiently transfected the expression vector for the virus receptor, pJET (1), into endometrial epithelial cells from rabbit, RBE7 (4), or human, Ishikawa (5), via the calcium phosphate precipitate technique. Transfection efficiencies of 5–10% were reached as determined by co-transfection of a RSV-LacZ vector followed by cytochemical staining for β -galactosidase activity. Following removal of the precipitate, 16 h after transfection, the cells were co-cultivated with the packaging cell line psi2 expressing v-Ha-ras and the neo gene as described (6). After 3–5 days of exposure to high virus titres (10^6 c.f.u./ml), the cells were selected for 4–6 weeks on G418 and the number of transformed clones was counted. Whereas in mock transfected cells no foci were detected, a large number of foci was generated in cells transfected with the murine ecotropic vector prior to infection. The number of foci obtained with 1×10^6 cells was in the range of 400 for RBE7 cells and 50 for Ishikawa cells. These transformation efficiencies are within the range obtained with a rat cell line of endometrial origin, RENT4 (6), infected with the same protocol. Southern blot analysis of five independent RBE7 foci demonstrated single copy retroviral integration (Fig. 1B). Homogeneous expression of the v-Ha-ras oncogene was demonstrated by immunofluorescence with anti Ha-ras antibodies, which decorated the cytoplasm only in transformed cells but not in control cells (Fig. 2).

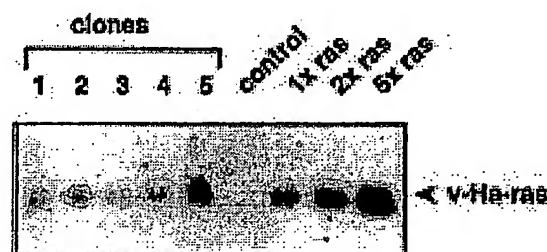


Figure 1. Southern blot analysis of integrated recombinant retrovirus sequences in isolated transformants. Genomic DNA (15 μ g) was digested with *Bam*HI, fractionated on a 1.2% agarose gel, transferred to nitrocellulose and hybridized with a 32 P-labelled v-Ha-ras probe (6). Lanes 1–5: DNA from independent G418 resistant transformants. Control and lanes 1x ras, 2x ras and 5x ras contain RBE7 DNA supplemented with 0, 35, 70 and 175 pg of Zip-ras-6 plasmid (7) respectively, corresponding to 0, 1, 2 and 5 copies per haploid genome.

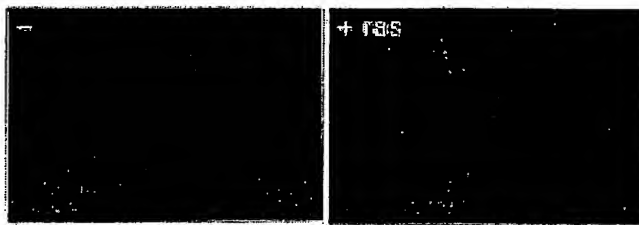


Figure 2. Immunofluorescence staining of a v-Ha-ras transformant clone (+ ras) compared with the uninfected RBE7 cell line (-). A combination of anti-v-Ha-ras monoclonal antibody (Dianova) and anti-rat-IgG FITC conjugate (Sigma) was used. V-Ha-ras expressing cells are uniformly stained, whereas RBE7 cells show only unspecific nuclear staining.

These results show that rabbit and human cells can be stably transformed with murine retrovirus as efficiently as rodent cells, simply by transient transfection of the murine ecotropic retrovirus receptor. This relatively simple and safe procedure could replace the hazardous use of amphotropic retroviral vectors and thus increase the safety of gene transfer procedures. This aspect could be

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particularly relevant in the context of human gene therapy as it would reduce the biological containment measurements required in hospital laboratories. Moreover, this technique could be used for the establishment of stable cell lines from primary cells of non-rodent origin. The basic principle could be extended to the use of other viral vectors which surface receptors have been cloned.

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Targeting lentiviral vectors to specific cell types *in vivo*

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Contributed by David Baltimore, June 15, 2006

We have developed an efficient method to target lentivirus-mediated gene transduction to a desired cell type. It involves incorporation of antibody and fusogenic protein as two distinct molecules into the lentiviral surface. The fusogen is constructed by modifying viral envelope proteins, so that they lack the ability to bind to their cognate receptor but still retain the ability to trigger pH-dependent membrane fusion. Thus, the specificity of such a lentiviral vector is solely determined by the antibody, which is chosen to recognize a specific surface antigen of the desired cell type. This specific binding then induces endocytosis of the surface antigen, bringing the lentivirus into an endosome. There, the fusogen responds to the low pH environment and mediates membrane fusion, allowing the virus core to enter the cytosol. Using CD20 as a target antigen for human B cells, we have demonstrated that this targeting strategy is effective both *in vitro* and in intact animals. This methodology is flexible and can be extended to other forms of cell type-specific recognition to mediate targeting. The only requirement is that the antibody (or other binding protein) must be endocytosed after interaction with its cell surface-binding determinant.

antibody | gene therapy | lentivirus | retrovirus | targeted gene delivery

Gene therapy is the introduction of a functional gene into a target cell to provide a therapeutic advantage (1). A particularly desirable gene therapy protocol would be to precisely deliver a gene of interest to specific cells or organs *in vivo* by means of administration of a designed gene delivery vehicle. Certain viruses are natural gene delivery systems, and much effort has been focused on engineering viral vectors as gene transfer vehicles (1, 2). Among these vectors, ones derived from oncoretroviruses and lentiviruses exhibit promising features because they have the ability to produce stable transduction, maintain long-term transgene expression and, for lentiviruses, to transduce nondividing cells. Targeting such viruses to particular cell types has proved to be challenging. We report here a general methodology that allows such targeting, even *in vivo*, and that is remarkably flexible.

Many attempts have been made to develop targetable transduction systems by using retroviral and lentiviral vectors (3, 4). Significant effort has been devoted to altering the envelope glycoprotein (Env), the protein that is responsible for binding the virus to cell surface receptors and for mediating entry. The plasticity of the surface domain of Env allows insertion of ligands, peptides and single-chain antibodies (5–14) that can direct the vectors to specific cell types. However, this manipulation adversely affects the fusion domain of Env, resulting in low viral titers. The unknown and delicate coupling mechanisms of binding and fusion make it extremely difficult to reconstitute fusion function once the surface domain of the same molecule has been altered (4). Another approach involves using a ligand protein or antibody as a bridge to attach the virus to specific cells (15–18). The challenge to this approach is that the Env, once complexed with the one end of the bridge molecule, fuses inefficiently. Because no practical strategies are available for targeted *in vivo* gene delivery, current gene therapy clinical trials are based on *in vitro* transduction of purified cells

followed by infusion of the modified cells into the patient. This is an expensive procedure, with significant safety challenges.

Our strategy involves uncoupling the target cell recognition function from the fusion function by providing them in separate proteins. For recognition, we use antibodies, and, for fusion, we use a viral glycoprotein that has been mutated to inactivate its binding ability. We make lentiviral vectors that incorporate both molecules into their surface. Our working hypothesis was that the antibody should recognize a molecular constituent on the target cell membrane and attach the lentivirus to the cell surface (Fig. 5, which is published as supporting information on the PNAS web site). Antibody binding should then induce endocytosis, bringing the lentivirus into an endosome. There, the fusogenic molecule (FM) should respond to the low pH environment and trigger membrane fusion, allowing the virus core to enter the cytosol. After reverse transcription and migration of the product to the nucleus, the genome of the vector should integrate into the target cell genome, incorporating the vector's transgene into the cell's inheritance.

Results

Construction of pH-Dependent Fusogen. Effective FMs for the proposed system should be able to incorporate into the lentivirus envelope and induce membrane fusion at low pH, independent of receptor binding. There are two classes of such FMs (19). The class I fusogens trigger membrane fusion using helical coiled-coil structures whereas the class II fusogens trigger fusion with β barrels. These two structures have different mechanics and kinetics (19), and both were evaluated to determine which would be better for the promotion of infection. One class I fusogen, HA from influenza A/fowl plague virus/Rostock/34 (FPV), was previously found to pseudotype murine leukemia virus (MLV) (20). Cannon and coworkers (21) created a binding defective version of FPV HA designated as HAMu (Fig. 1A). When incorporated into MLV displaying a functionally attenuated envelope glycoprotein, HAMu could enhance viral transduction efficiency (21). HAMu-mediated fusion is thought to be independent of receptor binding (3). The class II FM that we tested was the Sindbis virus glycoprotein from the alphavirus family (22) and is designated as SIN. SIN consists of two transmembrane proteins (23), one responsible for fusion (E1) and the other for cell binding (E2). SIN is known to pseudotype both oncoretroviruses and lentiviruses. By inserting the IgG-binding domain of protein A (ZZ domain) into the E2 protein and making several additional mutations to inactivate the receptor-binding sites, Chen and coworkers (16) made a binding-deficient and fusion-competent SIN. We adapted this form of SIN but replaced the ZZ domain with a 10-residue tag sequence, for which

Conflict of interest statement: No conflicts declared.

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Abbreviations: FM, fusogenic molecule; Env, envelope glycoprotein; SIN, Sindbis virus glycoprotein; α CD20, anti-human CD20 antibody; TU, transduction units; PBMC, peripheral blood mononuclear cells; FPV, influenza A/fowl plague virus/Rostock/34.

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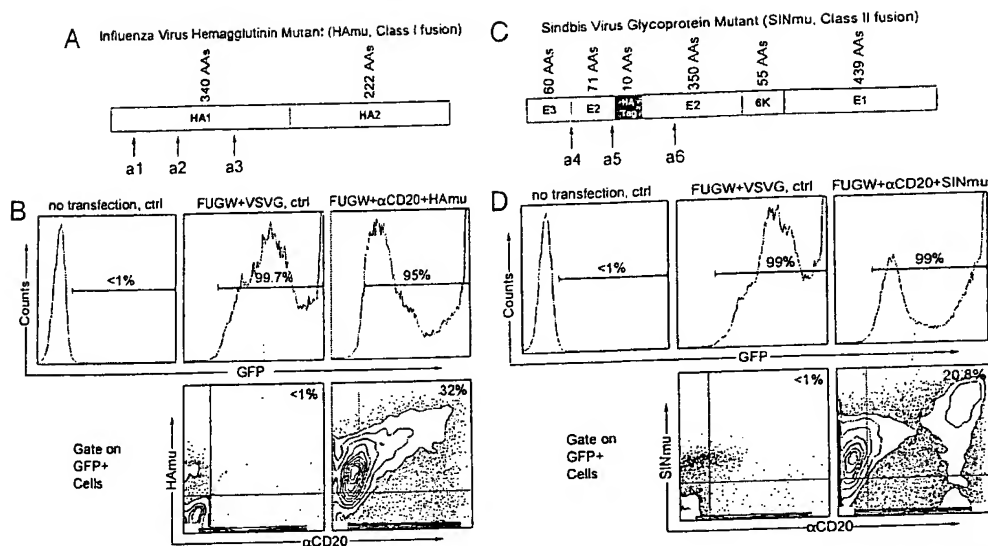


Fig. 1. Coexpression of antibody and fusogenic protein on the surface of the virus packaging cell line. (A) The class I fusion protein HAMu derived from influenza A (FPV) HA. HA contains two glycoproteins after maturation: HA1 for binding to cell surface receptor, sialic acid; HA2 for triggering membrane fusion. Three point mutations within the receptor binding sites (a1, Y106F; a2, E199Q; a3, G237K) (21) were introduced to generate the binding-defective but fusion-competent HAMu. Single letter amino acid abbreviations are as follows: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; K, lysine; M, methionine; P, proline; Q, glutamine; V, valine; Y, tyrosine. (B) FACS analysis of virus-producing cells. 293T cells were transiently transfected with plasmids encoding the P, proline; Q, glutamine; V, valine; Y, tyrosine. (C) The class II fusion protein SINmu derived from SIN. SIN contains two membrane glycoproteins (E1 and E2) and a signal peptide (E3). E1 for mediating fusion, E2 for receptor binding, and E3 as a signal sequence for processing of E2 glycoprotein. A 10-residue tag sequence (MYPYDVPDYA) was inserted between amino acids 71 and 74 of the E2 glycoprotein. A series of alterations (a4: deletion of amino acids 61–64 of E3; a5: mutations of 68SLKQ71 into 68AAAA71; mutations of 157KE158 into 157AA158) (16) was introduced to yield the binding-defective and fusion-competent SINmu. (D) Directly analogous to B, except that SINmu was used for the fusion protein and was detected by an anti-tag antibody.

there exists a monoclonal antibody that allows monitoring of SIN expression; we designated it SINmu (Fig. 1C).

Construction of Membrane-Bound Antibody for Targeting. The antibody that we have chosen for targeting in this study is the anti-human CD20 antibody (α CD20), a version of which is currently being used in the treatment of B cell lymphomas. We generated a construct that encodes a mouse/human chimeric anti-CD20 antibody with the human membrane-bound IgG constant region (α CD20). Genes encoding human Ig α and Ig β , the two associated proteins that are required for surface expression of antibodies, were cloned into a construct designated pIg $\alpha\beta$.

Preparation of Recombinant Lentiviral Vectors. The production of lentiviruses enveloped with both anti-CD20 antibody and the candidate FM was achieved by cotransfection of 293T cells with the lentiviral vector FUGW, plasmids encoding viral *gag*, *pol*, and *rev* genes, α CD20, pIg $\alpha\beta$ and pFM (the plasmid encoding a FM, either HAMu or SINmu), by using a standard calcium phosphate precipitation method (24). FUGW is a self-inactivating and replication-incompetent lentiviral vector that carries the human ubiquitin-C promoter driving a GFP reporter gene (25). As a control, the Env derived from vesicular stomatitis virus (VSVG) was used as a joint recognition and fusion protein. FACS analysis of the transfected cells showed that virtually all expressed some level of GFP as an indicator of the presence of the viral vector (Figs. 1B and D Upper). Some 30% of GFP-positive cells coexpressed HAMu and α CD20 on the cell surface (Fig. 1B Lower). A slightly smaller percentage (\approx 20%) of the 293T cells exhibited coexpression of GFP, SINmu, and α CD20 (Fig. 1D). The resultant viruses from

these transfected production cells were designated FUGW/ α CD20+HAMu and FUGW/ α CD20+SINmu.

Coincorporation of Fusogen and Antibody into Lentiviral Vectors. To examine whether α CD20 and the FM were incorporated in the same virion, we performed a virus–cell binding assay. As a target, we made a 293T cell line stably expressing the CD20 protein antigen (293T/CD20; Fig. 2A). The parental cell line 293T served as a negative control. The viral supernatants were incubated with the target cells at 4°C for half an hour. The resultant binding was assayed by means of a three-staining scheme (Fig. 2B). FACS analysis showed that lentivectors bearing α CD20 were in fact able to bind to CD20-expressing 293T cells (Fig. 2C Upper). The control of 293T cells with no CD20 expression displayed no detectable α CD20, showing that the virus binding to cells must be due to a specific interaction between the cell surface CD20 antigen and the viral surface α CD20 molecule. In another control, the virus bearing only FM exhibited no ability to bind either cell line, indicating that the HAMu and SINmu did lack the capacity for cell binding (L.Y., L.B., D.B., and P.W., unpublished work). FACS analysis also showed that the virus bound to the 293T/CD20 cell surface displayed the FMs (Fig. 2C Lower), suggesting that both α CD20 and FM were incorporated on the same virion, which was further confirmed by FACS plots of α CD20 versus FM (Fig. 2D). In addition to codisplay, these results suggest that the presence of the FM does not affect the α CD20 binding to CD20.

Targeted Transduction of Lentiviral Vectors to Cell Line *In Vitro*. We next examined whether α CD20-bearing virus can transfer genes into cells expressing CD20 in a cell-specific manner. GFP expression was used to measure the transduction efficiency. The

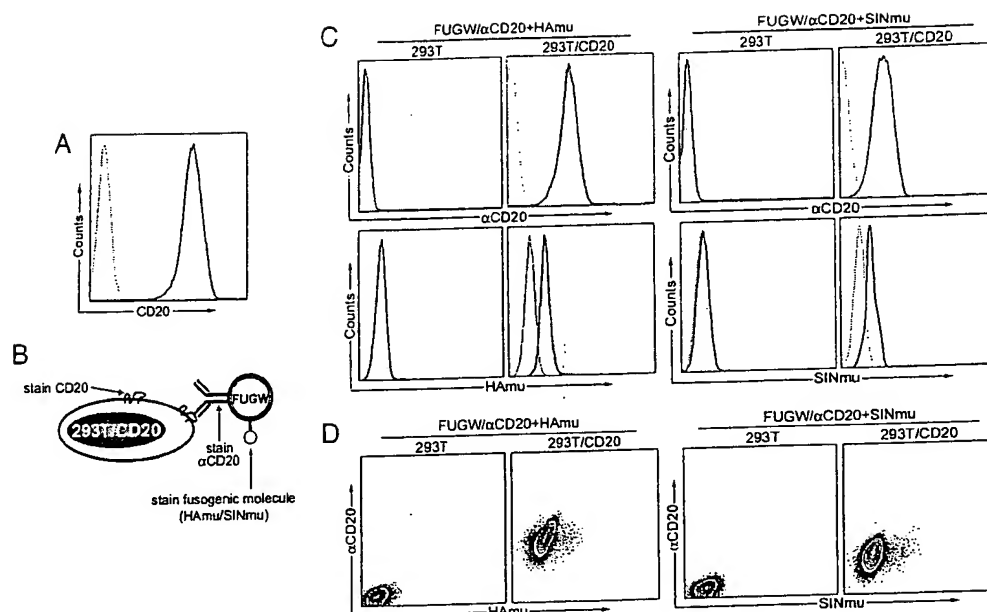


Fig. 2. Virus-cell binding assay to study the codisplay of antibody and fusogenic protein on the lentiviral surface. (A) FACS analysis of target cell line 293T/CD20. CD20 expression was detected by using anti-CD20 antibody. Solid line, expression of CD20 in 293T/CD20; shaded area, CD20 expression in 293T cells (as a control). (B) Schematic representation of three-staining scheme used for analyzing virus-cell binding assay. Three stainings were used to detect the presence of CD20, α CD20, and the fusogenic molecule (HAmu or SINmu), respectively. (C Left) FACS plots of 293T/CD20 cells incubated with FUGW/ α CD20+HAmu. The binding of virus to 293T/CD20 cells was detected by antibody against α CD20 (anti-IgG) and HAmu. Solid line, analysis on 293T/CD20; shaded area, analysis on 293T (as a control). (C Right) FACS plots of 293T/CD20 cells incubated with FUGW/ α CD20+SINmu. The binding of virus to 293T/CD20 cells was detected by antibody against α CD20 and SINmu. Solid line, analysis on 293T/CD20; shaded area, analysis on 293T (as a control). (D) Codisplay of antibody and fusogenic protein was analyzed by a density plot correlating the presence of the two proteins.

supernatants containing lentivectors bearing various surface proteins were incubated with CD20-expressing target cells, and 293T cells served as a control. Four days posttransduction, the efficiency of targeting was analyzed by FACS. Fig. 3A (rightmost image) shows that FUGW/ α CD20+HAmu viral particles could specifically transduce 16% of 293T/CD20 cells. Images to the left show that transduction required the presence on the virions of HAmu, but there was some background transduction with virions lacking α CD20, likely because of residual weak binding of HAmu to its ligand, sialic acid. The titer for FUGW/ α CD20+HAmu (fresh viral supernatant, no concentration) was estimated to be $\approx 1 \times 10^5$ transduction units (TU)/ml; the titer was determined by the percentage of GFP⁺ cells in the dilution ranges that showed a linear response. The 293T cells showed a small background infection level but no specific transduction by FUGW/ α CD20+HAmu (Fig. 3A Lower). When SINmu was used as the fusion protein, substantial enhancement of specific transduction was observed (52%; Fig. 3B). The titer for FUGW/ α CD20+SINmu was estimated to be $\approx 1 \times 10^6$ TU/ml. Also, we detected a much lower transduction in the absence of the binding protein ($\approx 1\%$). Thus, the data in Fig. 3B show that SINmu is a better fusion protein to partner with α CD20 for targeting lentiviral vectors. When we monitored the transduction at various time points using FACS, we found that SINmu-containing virions exhibited faster transduction kinetics than those with HAmu (L.Y., L.B., D.B., and P.W., unpublished work). Both FUGW/ α CD20+HAmu and FUGW/ α CD20+SINmu could be concentrated by ultracentrifugation with a >90% recovery rate, which is important for *in vivo* application.

To assess whether α CD20 and the fusion protein (HAmu or SINmu) had to be incorporated into the same viral particle, and

therefore functioned in *cis* to mediate transduction, we mixed FUGW/ α CD20 with FUGW/HAmu or FUGW/SINmu, each displaying only one protein, and tested their transduction of 293T/CD20 cells. This procedure did not result in specific transduction, indicating that the specific transduction conferred by the engineered recombinant viruses requires the two proteins to be displayed on the same viral particle.

Antibody-Antigen Interaction Responsible for Targeted Transduction. It seems that two distinct proteins can carry the binding and fusion events of engineered lentiviruses for targeted transduction. To further confirm that the specificity we observed was a consequence of interaction between α CD20 and CD20, we transduced 293T/CD20 cells in the presence of anti-CD20 blocking antibody. As expected, a dramatic decrease of infectivity was detected for both FUGW/ α CD20+HAmu (L.Y., L.B., D.B., and P.W., unpublished work) and FUGW/ α CD20+SINmu (Fig. 3D), suggesting the essential role of antibody-antigen binding for the targeted transduction.

Confirmation of pH Dependence of Fusogen. To examine the requirement for a low pH compartment to allow the recombinant lentivectors to penetrate into cells, we incubated both FUGW/ α CD20+HAmu and FUGW/ α CD20+SINmu with 293T/CD20 cells in the absence or presence of ammonium chloride (NH_4Cl), which can neutralize acidic endosomal compartments. Addition of NH_4Cl to cells completely abolished transduction by either FUGW/ α CD20+HAmu (L.Y., L.B., D.B., and P.W., unpublished work) or FUGW/ α CD20+SINmu (Fig. 3E). These results are consistent with the low pH requirement of HA and SIN to trigger membrane fusion. More direct evidence for pH-dependent fusion was provided by a cell-cell fusion assay. 293T cells expressing GFP,

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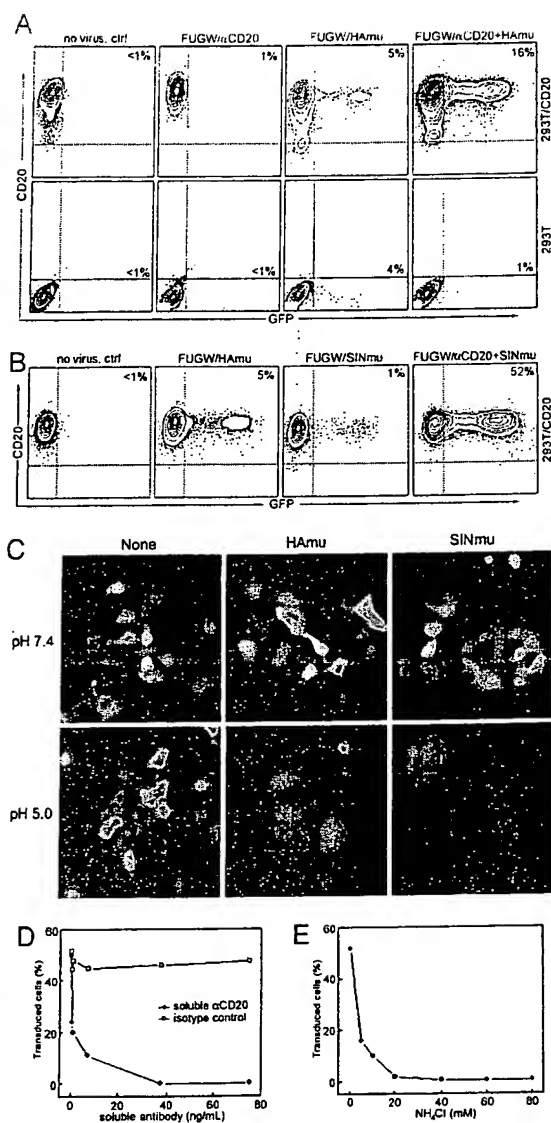


Fig. 3. Targeting of lentivectors bearing both antibody and fusion protein to 293T/CD20 cells *in vitro*. (A) 293T/CD20 cells (2×10^5) were transduced with 500 μ l of fresh unconcentrated FUGW/αCD20 (no HAMu), FUGW/HAMu (no αCD20), or FUGW/αCD20+HAMu. 293T cells (no expression of CD20) were included as controls. The resulting GFP expression was analyzed by FACS. The specific transduction titer for FUGW/αCD20+HAMu was estimated to be $\sim 1 \times 10^5$ TU/ml. (B) A similar transduction experiment was performed by using unconcentrated FUGW/SINmu (no αCD20) or FUGW/αCD20+SINmu. For comparison of targeting specificity, cells were also transduced with FUGW/HAMu. The specific transduction titer for FUGW/αCD20+SINmu was estimated to be $\sim 1 \times 10^6$ TU/ml. (C) Evidence of pH-dependent fusion of HAMu and SINmu by a cell-cell fusion assay. 293T cells (0.1×10^6) transiently transfected to express GFP and surface αCD20 and fusion protein (either HAMu or SINmu), and 293T/CD20 cells were mixed together, washed once with normal PBS (pH 7.4), and incubated in low pH PBS (pH 5.0) or normal pH PBS (as a control) for half an hour at 37°C. The cells were then washed and cultured in the regular medium for 1 day. Cells were visualized by epifluorescence microscope equipped with a GFP filter set. (D and E) Effect of addition of soluble αCD20 (D) or NH₄Cl (E). αCD20 or NH₄Cl was added into viral supernatants during transduction for 8 h. Then, the supernatants were replaced with fresh medium. The cells were analyzed for GFP expression after 2 days. Isotype-matched antibody was used as a control for D.

surface αCD20, and FM were incubated with 293T/CD20 cells in a low-pH buffer for half an hour, followed by culturing in regular medium. Both HAMu and SINmu induced cell-cell fusion by forming multinucleated polykaryons (Fig. 3C). The interaction between αCD20 and CD20 dramatically enhances the probability of fusion, because a similar experiment with cells that lacked αCD20 and CD20 yielded a much lower level of fusion (L. Y., L.B., D.B., and P.W., unpublished work). The αCD20/CD20 interaction probably brings the cell membranes into close apposition, facilitating the action of the fusion protein.

Targeted Transduction of Lentiviral Vectors to Primary B Cells. Having established the ability of the system to mediate CD20-specific transduction of artificially created cell lines, we next investigated the possibility of specific transduction of primary human B-lymphoid cells, cells that naturally carry the CD20 antigen. Fresh, unfractionated human peripheral blood mononuclear cells (PBMCs) were transduced with FUGW/αCD20+SINmu and then stimulated with LPS to expand the B cell population. Four days later, the cells were stained for CD19 (a B cell marker), CD20, and GFP expression (Fig. 4A). We found that >35% of cells were CD20⁺ B cells under our culture condition. When we gated on CD20⁺ B cells, the majority of them were GFP⁺. On the contrary, virtually no GFP⁺ cells were detected when we gated on CD20⁻ non-B cells, confirming that the transduction was strictly dependent on CD20 expression. In another control experiment, fresh PBMCs were transduced with FUGW/αCD20+SINmu followed by stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin to expand T cells. FACS analysis of these T cells showed no expression of GFP (Fig. 6, which is published as supporting information on the PNAS web site), confirming transduction specificity.

To demonstrate that the targeting method is not limited to the lentiviral vector FUGW, we evaluated two additional lentiviral vectors with different promoter configurations. Kohn and co-workers (26) have incorporated the Ig heavy chain enhancer (E_H) with associated matrix attachment regions into lentivectors carrying either the human cytomegalovirus (CMV) promoter (CCMV) or the murine phosphoglycerate kinase promoter (CPGK). We adapted these two lentiviral vectors into our system and prepared recombinant lentiviruses CCMV/αCD20+SINmu and CPGK/αCD20+SINmu. Transduction of PBMC-derived B cells with these viral supernatants exhibited results similar to those observed previously with FUGW (Fig. 4A).

Targeted Transduction of Recombinant Lentiviral Vectors *in Vivo*. The real test of this system is whether it will mediate specific transduction *in vivo*. For this purpose, we used a human PBMC xenograft in a mouse model. Fresh human PBMCs (100×10^6 per mouse) were transferred into irradiated immunodeficient RAG2^{-/-}γ_c^{-/-} mice through a tail vein injection. Engineered lentiviruses bearing αCD20 and SINmu were administered through the tail vein 6 h after human cell transfer. After 2 days, we collected the whole blood from these mice, and the cells were analyzed for surface antigens and GFP expression. Approximately 30–40% of the cells recovered from the mice were human T cells (CD3⁺), and ~ 0.1 – 0.3% were CD20⁺ human B cells (Fig. 4B). Three populations were analyzed for GFP expression: CD20⁺, CD3⁺, and CD20⁻CD3⁻. None of the cells harvested from mice injected with virus bearing a control antibody and SINmu (FUGW/b12+SINmu) showed evidence of GFP expression in any of the three populations (Fig. 4B). In contrast, GFP expression was observed in at least 40% of the CD20⁺ cells isolated from mice injected with FUGW/αCD20+SINmu whereas no transduction was detected in the other two populations.

Discussion

This demonstration of targeting efficient gene delivery vehicles strictly to the desired cell types *in vivo* greatly enhances the

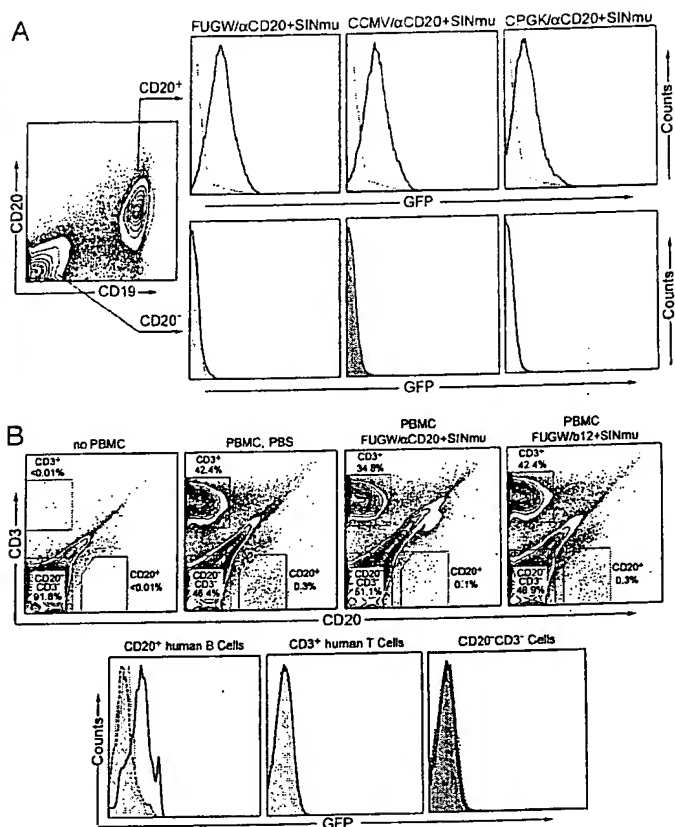


Fig. 4. Targeting CD20⁺ human primary B cells *in vitro* and *in vivo* using engineered lentivectors. (**A**) Fresh, unfractionated human PBMCs (2×10^6) were transduced by coculturing with concentrated FUGW/ α CD20+SINmu, CCMV/ α CD20+SINmu, or CPGK/ α CD20+SINmu (10×10^6 TU). LPS (50 μ g/ml) was added into the culture media for B cells to survive and grow. After 2 days, the B cell population was identified by costaining of CD19 and CD20. Solid line, analysis on transduced cells; shaded area, analysis on cells without transduction (as a control). (**B**) Fresh human PBMCs were transferred into irradiated RAG2^{-/-} γ c^{-/-} mice (100×10^6 per mouse) via tail vein injection. Six hours later, concentrated virus (100×10^6 TU per mouse) was injected through the tail vein. Two days later, whole blood was collected from these mice via heart puncture, and the cells were stained for human CD3 and CD20 and then analyzed by FACS for GFP expression. Shaded area, no virus treatment; dashed line, treated with FUGW/ α CD20+SINmu; solid line, treated with FUGW/ α CD20+SINmu.

therapeutic potential of lentivirus-mediated gene therapy and alleviates concerns of off-target effects. Possibly the most important implication of the work is that gene therapy could now be carried out as an inexpensive procedure, able to be considered even in the less-developed world.

In our approach, we break up the binding and fusion functions into two separate molecules that are inserted into the viral envelope. This methodology is particularly easy with lentiviruses (or other retroviruses) because these viruses readily incorporate into their envelope whatever proteins are found on the surface of producing cells (27). Other viruses have surfaces with many close-packed viral glycoproteins and exclude cellular proteins. A major advantage of this scheme over others where the viral protein is engineered with a foreign binding component is that the fusion protein maintains its full biological activity so that viral titer is not killed for increased specificity. The other key to the method is choosing a viral glycoprotein that mediates fusion in response to low pH and a cellular receptor that is efficiently endocytosed after antibody binding. The fusion molecule must exhibit fast enough kinetics that the viral contents can empty into the cytosol before the degradation of the viral particle. Our choice of CD20 as a target was arbitrary. We have already extended the method to other antibodies and cell surface receptor–ligand pairs.⁵ We envision that the flexibility (easy combination of

antibody, or other binding protein, and fusogenic molecule) and broadness (availability of monoclonal antibodies or ligands for many endocytosed cell-specific surface molecules) of this method will facilitate the application of targeted gene delivery for therapy and research.

Materials and Methods

Construct Preparation. The cDNAs of the human κ light chain constant region and the membrane bound human IgG1 constant region were amplified and inserted downstream of human CMV and EF1 α promoters, respectively, in the pBudCE4.1 vector (Invitrogen). We cloned the light and heavy chain variable regions from the murine anti-CD20 antibody (clone 2H7) using PCR amplification and inserted them directly upstream of the corresponding constant regions. The resulting construct was designated p α CD20. We cloned cDNAs of human Ig α and Ig β into the pBudCE4.1 vector (Invitrogen) to yield pIg $\alpha\beta$.

P. Cannon (University of Southern California and Childrens Hospital, Los Angeles) was kind enough to provide us with the construct encoding HAMu (21). We obtained the cDNA for wild-type SIN from J. Strauss's laboratory at the California Institute of Technology. PCR mutagenesis and assembly were used to generate the mutant SIN as described by Chen and colleagues (16), except a 10-residue tag sequence (MYPYDVPDYA) replaced the ZZ domain of protein A, which is located between amino acids 71 and 74 of the E2 glycoprotein of SIN. This version of SIN is designated SINmu.

Virus Production. Lentivectors were generated by transfecting 293T cells by using a standard calcium phosphate precipitation technique

⁵We have observed that this method can be exploited to target dendritic cells using a membrane-bound monoclonal antibody against the DEC-205 receptor. In addition, we found that incorporation of a membrane-bound form of stem cell factor could target c-kit-positive cells.

(24). 293T cells ($\approx 80\%$ confluent) in 6-cm culture dishes were transfected with the appropriate lentiviral vector plasmid (5 μg), together with 2.5 μg each of p αCD20 , p $\text{lg}\alpha\beta$, and the packaging vector plasmids (pMDLg/pRRE and pRSV-Rev) (28). The viral supernatants were harvested 48 and 72 h after transfection and filtered through a 0.45- μm pore size filter.

To prepare high-titer lentivectors, the viral supernatants were concentrated by using ultracentrifugation (Optima L-80 K preparative ultracentrifuge, Beckman Coulter) for 90 min at $50,000 \times g$. Particles were then resuspended in an appropriate volume of cold PBS.

Cell Line Construction. The 293T/CD20 cell line was generated by stable transduction via vesicular stomatitis virus (VSVG)-pseudotyped lentivector. The cDNA of human CD20 was cloned downstream of the human ubiquitin-C promoter in the lentivector plasmid FUW to generate FUW-CD20. The lentiviral vector FUW-CD20 was then pseudotyped with VSVG and was used to transduce 293T. The resulting cells were subjected to cell sorting to obtain a uniform population of CD20⁺ cells designated 293T/CD20.

Virus-Cell Binding Assay. Cells (293T/CD20 or 293T, 0.1×10^6) were incubated with 500 μl of viral supernatant at 4°C for half an hour and washed with 4 ml of cold PBS. The cells were then stained with the following three antibodies: an anti-human IgG antibody (BD PharMingen) to stain αCD20 , an anti-human CD20 antibody (BD PharMingen) to stain CD20, and an anti-FPV HA polyclonal antibody (obtained from H.-D. Klenk, Institute of Virology, Philipps University, Marburg, Germany) to stain HAMu, or an anti-tag antibody (Roche Applied Science, Mannheim, Germany) to stain SINmu. After staining, cells were analyzed by FACS analysis.

Targeted Transduction of 293T/CD20 Cells *in Vitro*. 293T/CD20 cells (0.2×10^6 per well) or 293T cells (0.2×10^6 per well) were plated in a 24-well culture dish and spin-infected with viral supernatants (0.5 ml per well) at 2,500 rpm, 30°C for 90 min by using a Beckman Allegra 6R centrifuge. Then, the medium was removed and replaced with fresh medium and incubated for a further 3 days at 37°C with $5\% \text{CO}_2$. The percentage of GFP⁺ cells was determined by FACS. The transduction titer was measured in dilution ranges that exhibited a linear response.

Effects of Soluble Antibody and NH_4Cl on Viral Transduction. 293T/CD20 cells (0.2×10^6) and 0.5 ml of viral supernatants were incubated for 8 h in the absence or presence of graded amounts of αCD20 (BD PharMingen) or NH_4Cl . The medium was replaced

with fresh medium and incubated for another 2 days at 37°C with $5\% \text{CO}_2$. FACS analysis was used to quantify transduction efficiency.

Cell-Cell Fusion Assay. 293T cells (0.1×10^6), transiently transfected to express GFP, surface αCD20 , and fusion protein (either HAMu or SINmu), and 293T/CD20 cells (0.1×10^6) were mixed together, washed twice with normal PBS (pH 7.4), and incubated in 150 μl of low pH PBS (pH 5.0) or normal pH PBS (pH 7.4) (as a control) for half an hour at 37°C with $5\% \text{CO}_2$. The cells were then washed extensively and cultured in the regular medium for 1 day. Cells were visualized by an epifluorescence microscope equipped with a GFP filter set.

Targeted Transduction of Primary Human B Cells *in Vitro*. Fresh, unfractionated human PBMCs (2×10^6) (AllCells) were incubated with concentrated virus with total TUs of 10×10^6 (based on the titer on 293T/CD20 cells). LPS (50 $\mu\text{g}/\text{ml}$) was then added for B cells to survive and grow. After 2 days, cells were harvested and washed in PBS. B cell population was determined by FACS staining using anti-human CD20 and CD19 antibodies. Targeting transduction was quantified by gating on the different populations of cells and measuring their GFP expression.

Targeted Transduction of Primary Human B Cells *in Vivo*. RAG2^{-/-} γ_c ^{-/-} female mice (Taconic) of 6–8 weeks old were given 360 rad whole-body irradiation. On the following day, 100×10^6 fresh human PBMCs (AllCells) were transferred by tail vein injection into each mouse. After 6 h, concentrated virus (100×10^6 TU per mouse) or PBS (as control) was administered via the tail vein. Two days later, whole blood was collected from these mice via heart puncture, and the cells were stained for human CD3 and CD20 and then analyzed by FACS for CD3, CD20, and GFP expression. The mice were maintained on the mixed antibiotic sulfamethoxazole and trimethoprim oral suspension (Hi-Tech Pharmacal) in a sterile environment in the California Institute of Technology animal facility in accordance with institute regulations.

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